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METABOLIC POLYMORPHISMS IN MAMMALS AND THEIR BEARING ON PROBLEMS OF BIOCHEMICAL GENETICS*

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Numerous chemical differences between members of a single mammalian species have come to light during the past few years. Most of these are known to be genetically determined by simple switch mechanisms. Since the alternative genes occur in populations at frequencies well above those explicable by estimated mutation rates, the differences represent metabolic polymorphisms. No firm distinction can be drawn between these and other polymorphisms, all of which must ultimately have a basis in chemical differences between gene products. Nevertheless, the classical structural and pigmentary polymorphisms (occurring, for example, in mimetic butterflies and variously pigmented and banded snails) produce clear overt differences between phenotypes which are known to affect susceptibility to predation. The selective effects of the polymorphisms to be described must be sought, on the other hand, in physiological differences between phenotypes which resemble one another closely in external appearance.

The metabolic polymorphisms in mammals have attracted interest for a number of reasons. First, they provide information about the chemical individuality of members of a species, which is of interest in itself as well as being responsible for such practically important phenomena as the reaction against homografts. Second, they show how the products of closely related genes can differ, and thus provide insight into the effects of mutation on the gene products. Third, selective agencies which have been found to act upon the several genotypes have helped to reveal the genetical mechanisms by which polymorphisms are maintained. Fourth, the frequencies of the genes concerned vary from race to race and breed to breed, and may serve as a guide to the remote ancestry of individual populations. And, fifth, metabolic polymorphisms provide marker loci on chromosomes in which markers for linkage studies are most needed, namely, the chromosomes of man and the larger domestic mammals.

*A preliminary communication of the main conclusions in this paper was presented to the Tenth International Congress of Genetics, Montreal, August, 1958.

AMINO ACID REPLACEMENTS

The simplest and most common situation seems to be that in which allelic genes control the formation of closely related but distinct proteins, two of which are present in heterozygotes. An example is the formation of normal adult human hemoglobin, sickle-cell hemoglobin and hemoglobin C, which are electrophoretically distinct. The differences between these hemoglobin types have been chemically defined by Ingram (1957) and Hunt and Ingram (1958). It appears that a single negatively charged amino-acid (glutamic acid) in each half-molecule of normal adult hemoglobin is replaced in sickle-cell hemoglobin by a neutral residue, valine, and in hemoglobin C by a positively charged residue, lysine. This small substitution has dramatic consequences: the change in overall charge allows the sickle-cell hemoglobin molecules to aggregate when deoxygenated and so brings about the sickling of erythrocytes, hemolysis and vascular obstruction characteristic of sickle-cell disease. Since it is likely that the sickle-cell and hemoglobin C genes have arisen as mutations from the normal allele, the effect of the mutations can be defined as the replacement of one amino-acid by another in the gene products. This could be the result of alteration of a single base pair in the DNA double helix.

Peptides and proteins from different species often differ by single amino-acid substitutions, and again it is reasonable to suppose that each of these arose by single mutational steps. Thus, Popenoe, Lawler and du Vigneaud (1952) found that vasopressin from beef pituitary glands contains arginine as one of its eight constituent amino-acids while in vasopressin from hog glands lysine is present instead of arginine, the remaining amino-acids being the same. Brown, Sanger and Kitai (1955) found that sheep insulin differs from beef insulin in the substitution of a glycine residue for serine at position 9 in the A (glycyl) chain. Tuppy and Paléus (1955) reported that ferriporphyrin-containing peptides derived from cytochrome C have the same composition in the pig, horse, cow and salmon; in the chicken a serine residue replaces alanine. And Li (1957) stated that bovine pituitary malanophore-stimulating hormone differs from porcine only at position 2, where a serine residue replaces glutamic acid. The latter is analogous to the hemoglobin mutations in that a neutral amino-acid residue replaces a charged one, and, as expected, the two hormones differ in electrophoretic mobility.

The examples which have been quoted represent minimal substitutions; in other cases more than one amino-acid is altered. Thus, the only differences in insulins from different species so far detected lie in the 8.9.10 sequence of residues within the intrachain disulphide bridge of the A chain (Harris, Sanger and Naughton, 1956; Ishihara, Saito, Ito and Fajino, 1958). The sequence is alanine.serine.valine in cattle, alanine.glycine.valine in sheep, threonine.serine.isoleucine in the pig and the sperm whale, threonine.glycine.isoleucine in the horse and alanine.serine.threonine in the sei whale. Whether these larger substitutions represent more than one mutational step, or a single step involving two or more DNA base pairs (possi-

bly a small intragenic inversion) is at present unknown. Incidentally, the mutability of insulin at the 8.9.10 positions in the A chain, and of normal adult human hemoglobin at one point, suggests that certain parts of the DNA within the genes controlling their synthesis may be less stable than others. It might be inferred that mutations affecting other parts of the insulin molecule would be lethal. This, however, does not necessarily follow since considerable manipulation of insulin has been achieved without loss of activity, for example, removal of C-terminal residues and acetylation of free amino groups. Different degrees of stability within one small section of the T4 coliphage chromosome, both normally and in the presence of mutagenic agents, have been shown by the studies of Benzer (1957).

HUMAN HEMOGLOBIN TYPES

Returning to the human hemoglobin polymorphism, it seems clear that in homozygotes only one product is formed (apart from fetal and minor hemoglobin components, which appear to be under independent genetic control). In heterozygotes both allelic genes are present and control the formation of the two hemoglobin types side by side in each red cell precursor. This can be concluded from the observation that there are not two classes of cell, only one of which becomes sickle-shaped on deoxygenation; all cells assume the sickle shape at oxygen tensions much lower than those required to induce sickling of the cells of homozygotes. Hence each red cell contains a mixture of normal adult and sickle-cell hemoglobin.

The rates of formation of the several hemoglobin types differ; normal adult human hemoglobin is synthesized more rapidly than sickle-cell hemoglobin or hemoglobin C (Itano, 1953). These appear to be absolute rather than relative rates of synthesis since the total rate of hemoglobin formation is less in persons homozygous for the sickle-cell and hemoglobin C genes than in normal homozygotes (Thomas, Motulsky and Walters, 1955). Furthermore, the rates of formation of the several hemoglobin types is subject to modification by genes at other loci (Neel, Wells and Itano, 1951; Allison, 1955). In the extreme case, the thalassemia gene—in families in which it is inherited independently of the sickle-cell gene—suppresses the formation of normal adult hemoglobin, so that in individuals heterozygous for both the thalassemia gene and the sickle-cell or hemoglobin C genes, circulating hemoglobin is largely or exclusively of the abnormal type (see Singer, Josephson, Singer, Heller and Zimmerman, 1957). Such patients may be clinically and biochemically indistinguishable from those homozygous for the abnormal hemoglobin genes. This is an example of dominance modification in man, a subject discussed by Allison and Blumberg (1958).

The highly selective action of the thalassemia gene in suppressing the formation of normal adult hemoglobin, but leaving intact the synthesis of proteins differing from it by single amino-acid substitutions, is remarkable. However, such specificity of modification has parallels elsewhere in genetics. Thus, in Neurospora twenty-five mutants affecting the enzyme tryptophan synthetase (which in the presence of pyridoxal phosphate catalyses

the coupling of indole and serine to give tryptophan) were analyzed by Suskind (1957). He reported that the independently inherited suppressors of these mutants showed great specificity; for instance, five occurrences of Su_2 all suppressed only td_2, whereas Su_3 suppressed td_3 and td_2 but not three other mutants tested.

Studies of the inheritance of abnormal human hemoglobins have raised other problems of general interest. Evidence has been presented that the genes responsible for the formation of hemoglobins N (formerly known as G) and Hopkins 2 (which may be equivalent to J) are not at the same locus as the sickle-cell gene (Schwartz, Spaet, Zuelzer, Neel, Robinson and Kaufman, 1957; Smith and Torbert, 1958). The findings imply that genes at different loci can influence the type of a single protein synthesized. This is difficult to reconcile with the template hypothesis in its original form, according to which one gene controls the formation of one protein type through the agency of one RNA template. Alternative hypotheses seem at present equally unattractive. The possibility that two widely separated pairs of genes control the synthesis of normal adult hemoglobin (in much the same way that an independent pair of genes seems to control the formation of the minor hemoglobin component, A_2, as described below) is unlikely, in view of the absence of normal adult hemoglobin in sickle-cell homozygotes. If genes at different loci control the formation of distinct precursor subunits of hemoglobin, small or large, they might be expected to combine at random, so that normal molecules as well as hybrid abnormal molecules would be formed in NS and JS heterozygotes. This has not been observed, so that if such subunits exist they must combine with one another in a non-random fashion.

Normal adult human subjects regularly show on starch gel electrophoresis a minor hemoglobin component, A_2. Ceppellini, Kunkel and Dunn (1958) have found a variant of this hemoglobin type which they have termed B_2. Heterozygotes show A_2 and B_2 in approximately equal proportions, the character segregating in families and having no influence on the major (A_1) hemoglobin component. The sickle-cell and hemoglobin C genes have no effect on myoglobin or fetal hemoglobin synthesis (Singer, Angelopoulos and Ramot, 1955; Schneider and Haggard, 1955). However, the identification in newborn children of two abnormal hemoglobin types which disappeared later (Fessas and Papaspyrou, 1957; Ager and Lehmann, 1958) suggests that genetically controlled variants of fetal hemoglobin may also exist, having no influence on the adult pigment. In human myoglobin, too, the fetal pigment appears to be distinct from the adult (Singer, Angelopoulos and Ramot, 1955).

A concept of general interest emerges. Independent genes exist controlling the formation of the related but distinct proteins, fetal and adult hemoglobin. In the course of development the synthesis of one product is suppressed and the other pair of genes takes over. This type of change may well apply to other proteins and be a major factor in embryonic differentiation.

The mechanism by which the sickle-cell polymorphism is maintained has been discussed by Allison (1957). Only a small proportion of sickle-cell homozygotes survive to reproductive age. The sickle-cell heterozygote is relatively resistant to malignant tertian malaria and has as much as a 25 per cent better chance of attaining adulthood than the normal homozygote in parts of Africa where malaria is hyperendemic. Thus the polymorphism is maintained by heterozygous advantage at a single locus. In West Africa an interesting complication is introduced into the picture by the presence of the hemoglobin C gene in high frequencies. Persons who inherit a sickle-cell gene from one parent and a hemoglobin C gene from the other are liable to a genetic variant of sickle-cell disease described by Kaplan, Zuelzer and Neel (1951). This condition is quite common in West Africa, more than one per cent of all children born in Ghana being liable to it. Because the sickle-cell and hemoglobin C genes are disadvantageous in combination, Allison (1955) postulated that the two must tend to be mutually exclusive in populations. This was subsequently found to be the case. Hemoglobin C is present in all West African territories, the incidence of heterozygotes rising to 29 per cent in Northern Ghana, and as the frequency of hemoglobin C increases that of the sickle-cell trait falls (Allison, 1956; Edington and Lehmann, 1956).

Thus powerful selective forces have stabilized the frequencies of the sickle-cell gene, the hemoglobin C gene and the normal allele in a complex genetic equilibrium. One heterozygote and the two abnormal homozygotes are at a disadvantage and the other two heterozygotes are apparently favored (Allison, 1956). The same sort of situation almost certainly obtains in other human populations in which genes for abnormal hemoglobins are common. Among many Mediterranean peoples the thalassemia trait has persisted in frequencies up to 20 per cent, although the homozygous condition is nearly always lethal. In several South-East Asian countries, such as Burma, Thailand and Indonesia, the genes for hemoglobin E, thalassemia and hemoglobin H are all common despite the fact that individuals who inherit two abnormal genes are liable to hemolytic disease. The most plausible inference is that the possession of a single abnormal gene is, or has been until very recently, advantageous, but the selective agencies involved have not yet been investigated.

HEMOGLOBIN TYPES IN OTHER MAMMALS

Polymorphisms of hemoglobin type are known also in cattle (Cabannes and Serain, 1955; Bangham, 1957; Bangham and Blumberg, 1958) and in sheep and goats (Harris and Warren, 1955; Evans, King, Cohen, Harris and Warren, 1956; Evans, Harris and Warren, 1958; van der Helm, van Vliet and Huisman, 1957). The B hemoglobin type in cattle is present in high frequencies in Jerseys, Guernseys and South Devons, but is absent from other British breeds. It is found in rather low frequencies in many French and Algerian breeds of cattle, and is relatively common in the Zebu cattle of Africa. Hemoglobin B is absent from the Muturu and N'Dama breeds of Ni-

geria, which are more susceptible to trypanosomiasis than Zebu cattle. Bangham and Blumberg (1958) suggest that the bovine B hemoglobin may possibly play a part in resistance against trypanosomes, but there is no direct evidence that this is so.

Evans and his colleagues have found that the frequency of the two sheep hemoglobin types varies considerably from breed to breed in Great Britain. The B hemoglobin type predominates in the lowland breeds, and A hemoglobin in the mountain and hill breeds. A clue to possible selective values of the sheep hemoglobin types has emerged from the work of Huisman, van Vliet and Sebens (1958) in Holland. These authors report that the two hemoglobins differ considerably in oxygen affinity. Type A (called type II by Huisman) has the greater affinity for oxygen, so that it might be more useful in acquiring oxygen from rarified air, and is in fact commoner than B in the mountain breeds. Still more remarkable is the claim of Huisman and his colleagues that when individual sheep of type AB were made anemic, production of hemoglobin B (type I) was favored at the expense of A. It was inferred that this change is adaptive, since, if the hemoglobin content of the blood were decreased because of anemia, it would be an advantage if the oxygen affinity of the hemoglobin were as low as possible, so that relatively more oxygen could be transferred to the tissues. Further observations on this interesting problem are required.

Several other proteins show genetically controlled differences analogous to the hemoglobin types. Two electrophoretically distinct types of Beta-lactoglobulin have been found in cow's milk; any one animal may produce one, Beta_A, the other Beta_B, or both types (Aschaffenburg and Drewry, 1955, 1957; Blumberg and Tombs, 1958). The latter have also reported the existence of two alpha-lactalbumin types in the milk of some African cattle. As in the hemoglobin genes, the allelic Beta-lactoglobulin genes differ in the rate at which their products are formed. Given equal yields of milk of comparable casein content, cattle homozygous for Lg^A produce almost twice as much Beta-lactoglobulin as those homozygous for Lg^B, while the output of heterozygotes falls about halfway between those of the two homozygotes.

The application of two-dimensional starch gel electrophoresis has revealed three distinct types of the Beta-globulin binding iron, transferrin, in human serum, apparently controlled by three allelic genes. Two products are present in heterozygotes (Smithies, 1957, 1958). Drs. H. Harris and E. Robson of the London Hospital Medical College kindly allow me to state that they have found two more human Beta-globulin variants under similar genetical control. Genetically controlled heterogeneity of Beta-globulins in cattle and sheep has also been reported (Ashton, 1957, 1958; Smithies and Hickman, 1958).

HYBRID SUBSTANCES

In all the situations so far described, animals heterozygous for allelic genes produce two distinct products, each indistinguishable from the prod-

uct in the corresponding homozygote. The two synthetic mechanisms are operating side by side, but independently, in each cell. However, cases have come to light in which the gene products in heterozygotes are hybrid in character, differing from those in either homozygote. In other words, there is collaboration between the synthetic mechanisms set in train by the two allelic genes. Most of the examples refer to blood group antigens in different species. Thus, Morgan and Watkins (1956) found that human ABO blood group antigens from cyst fluids of AB subjects could be precipitated completely by anti-A or anti-B sera. If antigens from A and B subjects were mixed, only the homologous antigen was precipitated by each serum. It appears, therefore, that in heterozygous AB individuals the A and B genes give rise to a molecular species which differs from that produced by either gene in the homozygous state. A similar situation obtains in the rabbit blood groups. One system (factors controlled by the *Hg* locus) is of particular interest because it has three alleles, all serologically detectable. Factor I is only present when the animal is heterozygous AD (Cohen, 1956). This interaction factor resembles the "hybrid substance" in doves described by Irwin (1947).

The blood group antigens are several stages removed from the genes themselves, presumably being formed by protein enzymes, and it is perhaps not surprising that such hybrid products should occur in heterozygotes. However, it has been claimed that a similar situation obtains in the human haptoglobins, a group of serum proteins that combine with hemoglobin. Smithies (1955) showed that human subjects can be classified into three groups on the basis of the haptoglobin pattern shown by starch gel electrophoresis of serum. In one group, subsequently named 1-1, a single haptoglobin with high mobility at pH 8.4 is found; in another (group 2-2) several slower-moving haptoglobin bands are observed. In the third group (1-2, occurring in persons heterozygous for the two genes, Hp^1 and Hp^2) several haptoglobin bands of intermediate mobility are seen. Bearn and Franklin (1958) and also Smithies (personal communication) have found that the haptoglobins in the three phenotypes differ in size as well as charge. In group 1-1 a single low molecular weight component is found, and in group 2-2 several bands of higher molecular weight. In group 1-2 a band corresponding to that in group 1-1 and several bands of intermediate molecular weight occur. Bearn and Franklin have concluded that the haptoglobins in the Hp^1/Hp^2 heterozygotes are hybrid in character, like the blood groups. Allison (1959) has made an alternative suggestion that the haptoglobin molecules produced in type 2-2 resemble those in type 1-1 except in charge and in the possession of a combining site. Hence in group 1-1 only monomers exist, whereas in type 2-2 there are stable polymers of various sizes. In the heterozygotes both molecular species are synthesized; these form mixed low polymers, intermediate in size and charge between the products in the two homozygotes, with some residual monomers. The protein molecules formed by the allelic genes are not themselves hybrid: each allele independently forms its own kind of protein.

Also relevant in this connection is the report of Filliti-Wurmser, Aubel-Lesure and Wurmser (1953) that the molecular weights of the blood-group agglutinins in OO, A_1A_1 and A_1O individuals were 170,000, 300,000 and 500,000, respectively. However, there is some difference of opinion whether the formation of the naturally occurring isoagglutinins is under direct genetical control (Wiener, 1951; Race and Sanger, 1958).

INCOMPLETELY DOMINANT GENES

The third type of metabolic polymorphism encountered is that in which one genotype has a particular substance in relatively large amounts while other genotypes have smaller amounts or lack it altogether. Heterozygotes usually have levels intermediate between the extremes encountered in homozygotes. As a rule, mutations which give rise to markedly reduced activities of particular enzymes are disadvantageous and hence remain rare in natural populations. Such mutations are responsible for most of the classical inherited metabolic abnormalities in man (such as alcaptonuria and congenital galactosemia) as well as those in Neurospora and bacteria, which have shed much light on intermediary metabolism. Occasionally, however, such mutations remain common in mammalian populations. About 15 per cent of rabbits of many different stocks lack the enzyme atropine esterase in their plasma and liver. According to Sawin and Glick (1943), the capacity to split atropine is inherited in the form of a gene (*As*) located on the same chromosome as the gene (*E*) for diffusion of black pigment in the fur. The character is incompletely dominant, since homozygotes (*As/As*) produce more enzyme than heterozygotes, while no activity at all can be detected in the other homozygotes (*as/as*). Hobbiger and Lessin (1955) have reported that atropine block in nerves is much briefer in animals having the esterase than in those lacking it. Animals without the enzyme are said also to be more susceptible to poisoning by ingestion of plants containing atropine and related drugs; hence they might be at some disadvantage in the wild state.

Allison, ap Rees and Burn (1957) reported that in several breeds of dogs individual animals are found with high and others with low levels of the enzyme catalase in erythrocytes. The difference seems to be controlled by a single pair of genes. In *C/C* homozygotes more enzyme is present than in heterozygotes, while the catalase activities of *c/c* homozygotes are very low, and might be due to other ferrohaem compounds in erythrocytes. The *c/c* animals show no obvious anemia or other abnormality (unlike human subjects, in which acatalasemia is reported to be associated with progressive oral gangrene by Takahara, 1952). Their blood is particularly liable to hemolysis *in vitro*, however, large differences in this respect having been described in dogs by Cruz and Baumgarten (1957).

The polymorphism of the *As*, *as* genes in rabbits and the *C*, *c* genes in dogs could be explained if it were advantageous to have enzyme activities intermediate between the two extremes, since the heterozygotes would then be favored. Alternatively, pleiotropic effects of the genes concerned may

have influenced their selective values in such a way that both pairs of genes have persisted even in inbred populations.

An interesting example of this situation occurs also in man. It had been known for some years that certain individuals, most commonly of Negro or East Indian ancestry, suffer from hemolysis when given therapeutic doses of the antimalarial compound, primaquine, and other drugs. A series of biochemical abnormalities have been described in the erythrocytes of susceptible subjects, the primary defect probably being a drastic reduction in activity of the enzyme glucose-6-phosphate-dehydrogenase (Carson, Flanagan, Ickes and Alving, 1956). Family studies suggest that, as a rule, affected individuals are males hemizygous or females homozygous for an abnormal gene located on the X chromosome, with female heterozygotes having enzyme activities usually below normal (Childs, Zinkham, Browne, Kimbro and Torbert, 1958). Affected subjects are also liable to hemolysis after eating fava beans; this is the disease known as "favism" or "Bagdad spring anemia" which is common in some Mediterranean and Hither Asian countries, and which has a significant mortality. It would therefore be expected that selection would have operated against the abnormal gene, despite which it remains common in certain Mediterranean and African populations. This interesting sex-linked polymorphism would repay further investigation. The possibility that the abnormal gene—like the sickle-cell gene—confers resistance against malaria needs examination.

Other metabolic polymorphisms, the biochemical background of which is imperfectly understood, may also be due to inherited differences in the activity of single enzymes. The most remarkable is that affecting the cation composition of sheep erythrocytes (Evans, 1954). In most breeds some animals are found with high sodium and low potassium in erythrocytes (LK), and other animals with low sodium and high potassium (HK). The inheritance is controlled by a simple genetic mechanism with the HK character recessive (Evans, King, Cohen, Harris and Warren, 1956). Although these types are inherited quite independently of the sheep hemoglobin types, the A hemoglobin and HK genes are both commonest in highland breeds and least common in lowland breeds (Evans, Harris and Warren, 1958). Two human characters, apparently inherited as recessives, may also be due to single enzyme deficiencies. One is the inability to metabolize beet pigment ingested and the other is the inability to excrete methanethiol in the urine after ingestion of asparagus (Allison and McWhirter, 1956).

The spectacular progress that has been made in the recognition and study of mammalian metabolic polymorphisms in the past five years is likely to continue indefinitely as refined methods of analysis are applied to more and more bodily constituents. Already an enormous amount of chemical variability has been revealed—and also antigenic variability, which will at some stage be amenable to chemical definition. The uniqueness of the individual's chemical constitution is reflected in the reaction against homografts, which are tolerated only between uniovular twins or among highly inbred animals (Medawar, 1958).

The study of metabolic polymorphisms has also shed light upon general problems of biochemical genetics which have not been resolved by work on fungi, yeasts, bacteria or viruses, despite the obvious advantages of the latter as material for analyzing the mechanisms by which genes control metabolism. It is remarkable that the first steps towards a solution of the problem of how related genes affect the structure of their protein products should have been taken using human material. The next steps will probably come from the use of bacterial or bacterial virus preparations. But it can safely be predicted that much more information of general interest will emerge from the study of mammalian metabolic polymorphisms during the next few years.

SUMMARY

Examples of chemical differences between different members of a single mammalian species are reviewed. The commonest situation seems to be that in which allelic genes control the formation of closely related but distinct proteins, two of which are present in heterozygotes. The proteins can differ by as little as single amino-acid substitutions in each molecule or part of a molecule. The synthetic mechanisms set in train by the allelic genes often operate at unequal rates, and may be differentially modified. In the case of blood group genes, the products in heterozygotes can be "hybrid" in character and different from those in either homozygotes; but there is no evidence that this is true of protein products. In another group of polymorphisms, different amounts of the same product (usually enzymes) are present in the several phenotypes.

In some metabolic polymorphisms, notably the human hemoglobins, selective agencies are known to operate unequally on the different genotypes, maintaining the polymorphism through heterozygous advantage. There is suggestive evidence that other metabolic polymorphisms may also be subject to selection.

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SUGGESTED LITERATURE FOR STUDENTS IN PLANT BREEDING AND GENETICS*

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INTRODUCTION

It seems of importance to us that the student of today become familiar with important researches that are basic to present-day viewpoints. These readings have been selected because they seemed to the writers to be of particular value for this purpose.

While other papers would be of great value also, the ones cited should be of interest to all students of plant genetics, and will furnish a sound background of information.

East, E. M.

1907 The relation of certain biological principles to plant breeding. Conn. Agr. Exp. Sta. Bull. 158.

This is an admirable discussion of the biological work of Lamarck, Darwin, Weissmann, DeVries, Johannsen, Mendel and others. The principles elucidated are discussed in relation to the work of Vilmorin and others on sugar beets, that of LeCouteur, Shireff and Nilsson with small grains, and to hybridization studies of Kölreuter and Knight. The Knight-Darwin so-called law that "nature abhors perpetual self-fertilization" is discussed in relation to the breeding of cross-pollinated plants. Mendel's work is extensively reviewed in relation to its plant breeding applications. Vilmorin's isolation principle, that the only method of knowing the heritable characters of a plant is to grow and examine its progeny, is emphasized. This bulletin furnishes a valuable background for the study of original papers.

Vilmorin, Louis de

1856 Note sur la création d'une nouvelle race de betterave à sucre. pp. 25-29. In Notices sur l'amélioration des plantes by Louis Lévêque de Vilmorin and André Lévêque de Vilmorin. Vilmorin-Andrieux, Paris.

Probably this note is the original statement of Vilmorin's isolation principle mentioned above.

Darwin, Charles

1868[1] The variation of animals and plants under domestication. Vol. I, II.

*Contribution from the Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, Minnesota. Paper No. 982, Miscellaneous Journal Series, Minnesota Agr. Exp. Sta.

[1]The original dates of publication are given here, the date of the published translation follows the name of the publisher.

D. Appleton & Co., New York. 1900. First edition 1868, second 1875, third 1890.

1876 Cross and self-fertilization in the vegetable kingdom. D. Appleton & Co., New York.

Chapters 1 and 12 give the plans of the experiments and the results. It is very interesting to note the experimental methods used by Darwin at this early date. These books give an excellent idea of the status of ideas of variability and inheritance prior to the rediscovery of Mendel's Laws of Inheritance in 1900.

Weismann, A. The Germ-plasm, a Theory of Heredity.

1893 English translation (477 pages, indexed) by W. N. Parker and Harriet Rönnfeldt. Scribner's Sons, New York. 1898.

This is an interesting account of what was known at that time, and of Weismann's development of a theory of heredity and of the continuity of the germ plasm. Weismann reviewed the knowledge on chromosomes, development, hybridization, and the various kinds of reproduction known. The titles of the four parts in the book are: 1. The material basis of heredity, 2. Heredity in its relation to monogonic reproduction, 3. The phenomena of heredity resulting from sexual reproduction, 4. The transformation of species.

Vries, Hugo de

1901[1] The Mutation Theory. Vols. I, II. Translated by Farmer and Darbishire. The Open Court Publishing Co., Chicago. 1910.

A detailed presentation of the author's studies of mutation.

1906 Species and varieties, their origin by mutation. Lectures delivered by DeVries at the University of California. The Open Court Publishing Co., London.

In the preface the author writes, "My work claims to be in full accord with the principles laid down by Darwin, and to give a thorough and sharp analysis of some of the ideas of variability, inheritance, selection and mutation, which were necessarily vague at his time."

DeVries presents in a clear and interesting manner the results of his many experiments in relation to the above principles. Attention is called to the fact that most of DeVries' mutations were different from the changes to which the term is now applied.

The student will enjoy paging through the two 1901 volumes but may prefer to read more carefully the California lectures.

Scott, W. B.

1917 The Theory of Evolution. The Macmillan Co., New York.

This book of only 172 pages presents in non-technical language an outline of the available evidence for the truth of the evolutionary doctrine, including evidence from domestication, comparative anatomy, embryology, blood tests, paleontology, distribution and experiments.

Lull, R. S.

1917 Organic Evolution. The Macmillan Co., New York.

This textbook was written by the late well-known paleontologist when Professor of Vertebrate Paleontology at Yale University and Curator of the Peabody Museum. It is based largely on 23 years of college teaching. It presents the author's viewpoints with particular reference to paleontological evidence of evolution. Evidence on the mechanisms of evolution and evolution dynamics came at a much later date. For one of the first considerations of these aspects see Shull, A. F., Evolution. McGraw-Hill Book Co., New York. 1936.

Johannsen, W. L.

1903 Über Erblichkeit in Populationen und in reinen Linien. Gustav Fischer, Jena. (68 pages).

1909 Elemente der exakten Erblichkeitslehre. Gustav Fischer, Jena.

These books present the studies on which Johannsen based the pure line theory. They show that continuous selection in a homozygous line is of little or no value. In the 1909 book, Johannsen described several mutations that occurred in his pure lines.

Newman, L. H.

1912 Plant Breeding in Scandinavia. The Canadian Seed Growers Association, Canadian Building, Ottawa.

This summary is an excellent presentation of the methods and principles that were used at Svalöf in plant improvement.

The results of early studies of selection in cereals are reviewed and a clear presentation of later studies of hybridization as a means of improvement are reviewed in an interesting and readable manner. Studies with forages and potatoes also are summarized.

The work of Hjalmar Nilsson in 1891 is of considerable interest. At that time the breeders were making selections in cereals that were botanically or morphologically alike. Each type of selection was then grown in a separate plot. In some cases the plot consisted of the progeny of a single plant. At harvest some plots were remarkably uniform and these were then discovered to consist of the progeny of a single plant. This led in subsequent years to a study of single plant progenies as, "the quickest way, if not the only way, to obtain a uniform sort." Newman points out that this method had previously been used by Vilmorin in France where it had been called the "Vilmorin System of Selection." At Svalöf it was referred to as the System of Pedigree or Separate Culture. It appears to be the discovery of what later was called Johannsen's "Pure Line Theory."

Naudin, Ch.

1865 Nouvelles recherches sur l'hybridité dans les végétaux. Nouvelles Archives du Muséum d'Histoire Naturelle de Paris I: 25-176.

Naudin's results were explained by the segregation of specific substances

in the pollen and ovaries of the hybrid. He so closely approached the law later laid down by Mendel that some workers have spoken of it as the Naudin-Mendel law. His concept is similar to that developed later to explain the inheritance of quantitative characters.

Mendel, G. J.

1865 Versuche über Pflanzen-Hybriden. The Journal of Heredity Vol. 42, 1951. (Available as a separate reprint.)

This is a reprint of Mendel's original paper exactly as it appeared in the Verhandlungen Naturforschender Verein, Brunn Vol. 4, pp. 3-47. It was presented in February and March meetings in 1865. An English translation was printed in the Journal of the Royal Horticultural Society in 1901. Bateson published the translation separately in 1902 with a brief summary of Mendelism, entitled "Mendel's Principles of Heredity: A Defense." A reprint of the translation is now available from Harvard University Press, Cambridge.

Vries, Hugo de

1900 Sur la Loi de disjonction des hybrides. Comptes Rendus de l'Academie des Sciences (Paris) 130: 845-847.

Correns, C.

1900 G. Mendel's Regel Über das Verhalten der Nachkommenschaft der Rassenbastarde. Berichte der Deutschen Botanischen Gessellschaft 18: 158-168.

Tschermak, E.

1900 Über künstliche Kreuzung bei *Pisum sativum*. Berichte der Deutsche Botanischen Gessellschaft 18: 232-239.

The above three papers are available in English translation in Genetics, Number 5, Part 2, Volume 35: 1-47. 1950. This supplement also contains English translations of Mendel's letters to Carl Nägeli, 1866-1873.

The papers by DeVries, Correns and Tschermak report results from crossing experiments which parallel those obtained by Mendel in the 1860's. Each thought he had discovered something new. Only Correns and Tschermak cite and discuss Mendel's paper.[1]

The letters from Mendel to Nägeli were first published in 1905. They describe Mendel's experiments with other species many of which gave results similar to those with peas and also those with Hieracium species (many apomictic) with which Nägeli was working. Possibly it was because these latter did not agree that Mendel failed to publish his results with other species as a series of papers. Mendel did publish a second paper, Über einige aus künstlicher Befruchtung gewonnene Hieracium Bastarde, 1869, trans-

[1] For a full account of the discoveries of Mendel's paper, see Roberts, H. F., 1929, Plant Hybridization before Mendel. Princeton University Press, Princeton, N. J. DeVries discussed Mendel's paper in the first paper he wrote, but his second paper was published first.

lated in W. Bateson, "Mendel's Principles of Heredity." Cambridge University Press. 1909.

Spillman, W. J.

1902 Quantitative studies on the transmission of parental characters to hybrid offspring. U. S. Dept. of Agr. Office of Exp. Sta. Bull. 115: 86-101.

Spillman at Washington State about 1902 is considered by many to have independently rediscovered Mendel's Laws. He studied 14 different wheat variety crosses. Reciprocal crosses were alike in F_1. He wrote, "In every case, but one, which is noted later, the forms in each plot were simply combinations of the characters of the parental forms." He also wrote, "The types that tend to occur in the second generation include all possible combinations of the characters of the two parents."

Sutton, W. S.

1903 The Chromosomes in Heredity. Biological Bulletin 4: 231-251.

This paper by Sutton was an early clear statement of the parallelism between Mendelian segregation and the behavior of the chromosomes in reduction division and fertilization. Sutton says, for example, "Thus the phenomenon of germ cell division and of heredity are seen to have the same essential features."

Bateson, W.

1894 Materials for the Study of Variation. London.

1909 Mendel's Principles of Heredity. Cambridge University Press, London. 1909.

The purpose of the latter book, "is to give a succinct account of discoveries of heredity made by the application of Mendel's method of research." It contains a translation of Mendel's two papers together with a brief biographical note. The first chapter discusses pre-Mendelian writings and Mendel's methods and results. It then presents large numbers of cases of simple Mendelian inheritance in plants and animals including man. This is followed by a statistical discussion of the results and numerous cases of factor interaction to explain the inheritance of compound characters and other deviations from a 3 : 1 ratio.

R. C. Punnett joined Bateson as a co-worker in 1904. Bateson, Saunders and Punnett reported the first case of genetic linkage, which they termed "partial coupling," between flower color and pollen shape, and showed that the results in F_2 closely agreed with what would be expected from a system that produced gametes in the series 7BL : 1Bl : 1bL : 7bl. Such cases later were explained by the linear relation of genes in the chromosome.

Janssens, F. A.

1909 La théorie de la chiasmatypie. La Cellule 25: 389-411.

This paper calls attention to Weismann's theory, the agreement of Mendel's results with that theory, and also summarizes the information on cyto-

logical studies of meiosis. He then shows where the current theories failed to explain certain of the facts and presents his scheme of exchange between two of the four filaments of a chromosome pair. His concluding statement is that "it opens the field for a greater application of cytology to the theory of Mendel."

Sturtevant, A. H.

1913 The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association. Jour. Exp. Zoology 14: 43-59.

Six sex-linked factors in Drosophila were arranged in a linear series, using the number of crossovers per 100 gametes as an index of distance between any two factors.

The effect of double crossing over on observed distance and the effect of crossing over in one region on that in another are demonstrated. In conclusion, he states that the linear order of the factors forms a new argument in favor of the chromosome view of inheritance. It is one of the early and more interesting demonstrations supporting this view.

Morgan, T. H., A. H. Sturtevant, H. J. Muller and C. B. Bridges

1915 The Mechanism of Mendelian Heredity. Henry Holt & Co., New York.

This presents in a readable form certain of the early studies that led to the hypothesis that the genes are carried in a linear manner on the chromosomes and that this is the mechanism responsible for recombination of characters. The chapter on multiple factors is of special interest also. It contains a review of Castle's and Phillips' selection experiments with hooded rats that Castle at one time thought could be best explained on the basis that selection could modify a unit character. Later the results were explained, as had been done earlier with plants, by the action of multiple factors, in this case modifying factors that affected the hooded character.

As stated by Morgan in the preface, the purpose of the book was to "separate those questions that seem to us significant from that which is special or merely technical," hoping to bring about a better understanding between geneticists and workers in the traditional fields of zoology and botany.

Shull, G. H.

1908 The composition of a field of maize. Rept. Amer. Breeders Asso. 4: 296-301.

1909 A pure-line method in corn breeding. Ibid. 5: 51-59.

1910 Hybridization methods in corn breeding. Amer. Breeders Mag. 1: 98-107.

1911 The genotypes of maize. Amer. Nat. 45: 234-252.

1914 Duplicate genes for capsule form in *Bursa bursa-pastoris*. Zeit. indukt. abstamm. u. Vererbungsl. 12: 97-149.

The 1914 paper is of general interest because the term, "heterosis," is first suggested. It also presents Shull's work on duplicate factors. This latter work follows that of Nilsson-Ehle in 1908 and that of East in 1910 on

quantitative inheritance. Other papers in this field were published during this period but these referred to seem of greatest general interest.

All students of plant genetics will benefit from reading these early papers on inbreeding and cross-breeding. Shull (1952) has summarized his earlier concepts and his studies of self- and cross-fertilization in a paper entitled "Beginnings of the Heterosis Concept" published in "Heterosis," pp. 14-48, Iowa State College Press, Ames, Iowa, 1952.

East, E. M.

1908 Inbreeding in corn. Rept. Conn. Agr. Exp. Sta. for 1907, pp. 419-428.

East, E. M., and H. K. Hayes

1912 Heterozygosis in evolution and in plant breeding. U. S. Dept. Agr. Bur. Plant Industry Bull. 243.

East, E. M., and D. F. Jones

1919 Inbreeding and Outbreeding: their genetic and sociological significance. J. B. Lippincott Co., Philadelphia and London.

These three references present much of the experimental evidence with plants on the effects of inbreeding and the development of the heterosis concept. While Shull first suggested that pure lines should be developed in maize and that their crosses should be of commercial value, the work of East and co-workers was of equal or greater importance in relation to the development of breeding methods. Thus Wallace and Brown in their book, "Corn and Its Early Fathers," Michigan University Press, 1956, say, "We believe that priority in pointing out the importance of inbreeding as a technic in corn improvement belongs to Shull, but we also feel that corn breeders have been influenced more extensively by Edward Murray East." In the 1912 paper, East suggested the extensive use of hybrid vigor in crop plants and its importance in evolution. The student should appreciate the fact that each major discovery credited to a particular individual often had been discovered previously but not always utilized because of a lack of appreciation of its usefulness. Zirkle's 1952 paper, "Early Ideas on Inbreeding and Cross-breeding," in "Heterosis," pp. 1-12, illustrates this point. His seven summary statements of the knowledge of hybrid vigor at the beginning of the 20th century seem to cover most of the facts rediscovered and emphasized by East and Shull.

East, E. M.

1910 A Mendelian interpretation of variation that is apparently continuous. Amer. Nat. 44: 65-82.

A Mendelian explanation of quantitative inheritance is suggested based on work with corn and Nilsson-Ehle's studies with small grains. East had completed his explanation prior to his knowledge of Nilsson-Ehle's work. The data used by East are presented in greater detail by East and Hayes in "Inheritance in Maize," Conn. Agr. Exp. Sta. Bull. 167. 1911.

Morgan, T. H.

1914 Heredity and Sex. Columbia University Press, New York.

This excellent series of lectures discussed the evolution of sex and the mechanism of sex determination as known at that time, Mendelian principles of heredity and their bearing on sex, the effects of inbreeding and cross-breeding and other related problems.

Of special interest may be the chapter on fertility which discusses the work of Darwin on cross- and self-fertilization, that of Weissman concerning the effects of mixing germ plasms of two individuals, several studies on the effects of inbreeding in animals, the work of East and Shull with corn, and the various theories for the explanation of heterosis. A quotation from the discussion of the theories of Bruce and Keeble and Pellew to explain heterosis seems of special interest. Morgan says, "On this view the hybrid is vigorous, not because it is hybridous so to speak, but because in its formation a larger number of dominant factors (than were present in either parent) have been brought together." He also emphasizes the fact that these factors in the hybrid may be present in either a heterozygous or homozygous condition.

Winge, Ö.

1917 The chromosomes. Their number and general significance. C. R. Lab. Carlsberg 13: 131-275.

Winge presents early studies and hypotheses regarding the development of autoploids and amphiploids in the plant kingdom where ploidy in its various forms has played such an important role in species formation.

Emerson, R. A.

1914 Genetical studies of variegated pericarp in maize. Amer. Nat. 48: 87-115.

1917 The inheritance of a recurring somatic variation in variegated ears of maize. Genetics 2: 1-35.

Hayes, H. K.

1917 Inheritance of a mosaic pericarp pattern color of maize. Genetics 2: 261-281.

The above papers include reviews of earlier studies of variegation and mention the work of East and Hayes 1911 on inheritance in maize (Conn. Agr. Exp. Sta. Bull. 167), which contained an earlier study of pericarp variation and variegation.

Emerson concludes that there is a series of at least nine or ten multiple allelomorphs to which variegation belongs, some in the series highly stable, others frequently mutable.

Hayes carried on selection under conditions of self-pollination and isolated races breeding comparatively true for self red, variegated, very slight pattern and colorless pericarp. Selection within the selfed lines of varie-

gated pericarp lines modified the degree of variegation constantly, but did not lead to the development of a less variable progeny. Crosses between the four major types segregated on a monohybrid factor basis.

The crosses between the variegated race and the pattern color, "causes some sort of contamination which produces a condition of instability." From this cross also bud sports frequently were produced in the progeny. The conclusion is reached that the four major pericarp colors worked with form a series of multiple allelomorphs. The suggestion is made that certain combinations in the heterozygous condition produce germinal instability.

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A CICHLID FISH, *Symphysodon discus*, WITH UNIQUE NURTURE HABITS

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Although the Amazonian discus fish, *Symphysodon discus*, was first introduced to aquarists as long ago as 1933, extensive study of the breeding and life cycle of this spectacular species have only been recently pursued. Several detailed accounts of the breeding habits and rearing of these fish have appeared in the popular literature (Lindaman, 1953; Gordon, 1957; Skipper and Skipper, 1956a,b; 1957; Wolfsheimer, 1957). This paper will deal mainly with those aspects of the life cycle pertinent to the nurture of the fry.

Gustave Armbruster of Philadelphia was apparently the first to successfully breed *Symphysodon discus* in the spring of 1935. Adhesive eggs were deposited on a slate slab in a large aquarium with the water maintained at 85°F. and pH 6.8. The eggs were transferred to an aerated tank containing water of the same temperature and pH. The young began to hatch after two days and the yolk sac was completely absorbed after another three days. Infusoria, principally Hydatina, sieved through bolting cloth, were fed. At least half of the young died and growth for the first week was very slow. When the fry were two weeks old, finely sieved Daphnia were fed and growth became more rapid, but only 47 fish grew to maturity (Lindaman, 1953). Small numbers of young were subsequently reared from large spawnings by others, but in all instances the eggs were removed from the parents and nothing was learned of the offspring-parent relationship.

The first clue to the normal nurture came in 1949 when W. T. Dodd of Portland, Oregon, reported to the Oregon Aquarium Society that "the babies hung against the sides of the parents, receiving free-rides—using the breeders as landing fields." But since *discus* was usually difficult to breed and very expensive (even now a pair of adult breeders is worth about $350), other aquarists were unwilling to risk leaving the young with the parents. Yet even in the presence of an abundance of various aquatic microorganisms, few or no fry survived for more than a week in the absence of the parents. In 1955, Lois Saphian of St. Louis wrote to Gene Wolfsheimer of Sherman Oaks, California: "Of the last three spawnings, numbers 18 and 19 are thriving beautifully with very few losses. Number 20 will hatch tomorrow. I know now that the problem isn't food. They are eating newly hatched brine shimp with no trouble at all . . . all I reared were removed as soon as possible after spawning was completed. Every time I left the eggs in, they were eaten as they were hatching."

Other expert aquarists, notably Gene Wolfsheimer, Carrol Friswold, and Roy Skipper of England, were unable to induce the fry to eat any kind of

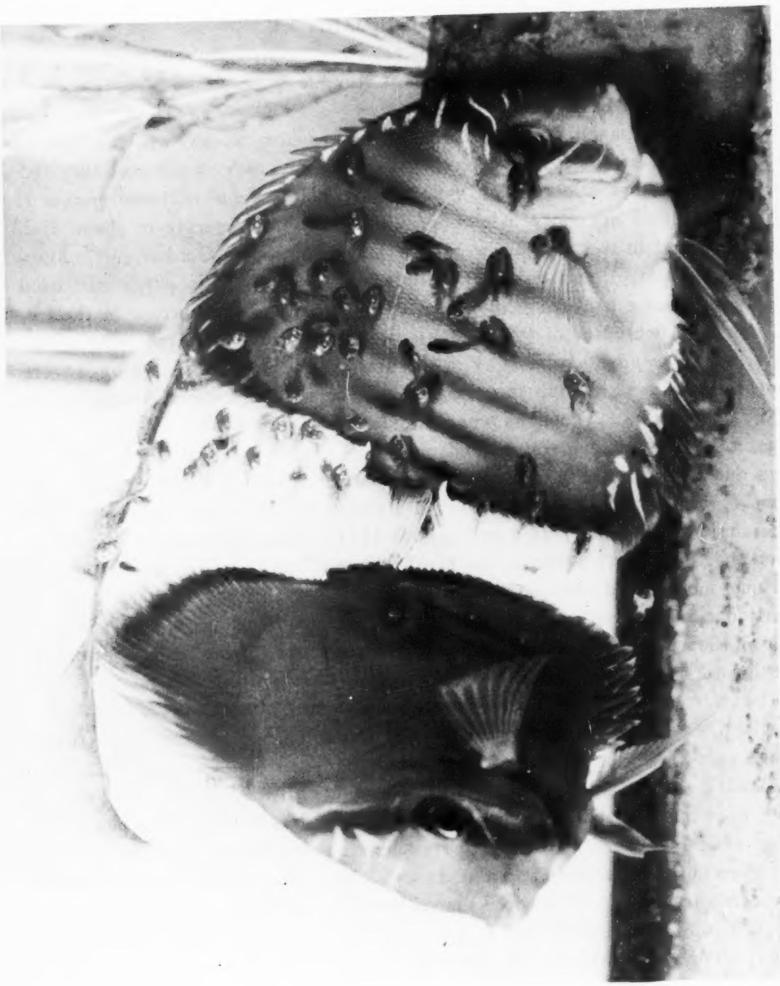

PLATE I. Fry of *S. discus* moving from one parent to the other. Photograph by G. Wolfsheimer.

PLATE II. Close-up view of fry of *S. discus* feeding off skin of one parent. Photograph by G. Wolfsheimer.

live food. The latter tried many different pure and mixed cultures of algae, protozoans, rotifers, etc., with separate groups of fry, but the result was always the same—all died of apparent starvation. This happened even when the newly-hatched fry were provided with changes of water from the tank containing the parents. Pairs of breeding adults from whom spawnings were removed continued to spawn regularly at intervals of about six days for many weeks. Ultimately, the adult pairs were given a chance to care for the eggs and young. Their characteristic behavior has now been repeatedly observed by various aquarists.

NORMAL PARENT-OFFSPRING RELATIONSHIP

Both parents take turns guarding, fanning, and mouthing the eggs. The parents pick up the newly-hatched fish with their mouths and transfer them together to various surfaces where each remains attached, wriggling violently at the end of a short thread. The fry become free-swimming four days after hatching and promptly move to their parents' sides where they begin to feed from their skin. Although both parents are capable of feeding the young, both take rest periods and, by a flick of the body, are proficient at transferring all the fry to the other parent. Alternatively, when there appears to be a scarcity of food on one of the parents, the fry will move to the other (see Plates I and II). After a week or more of feeding off the parents the fry will ingest other food such as newly-hatched Artemia or sifted nauplii of Cyclops. The young continue to feed on the parental skin for at least five weeks, even though an abundance of other live food is available (Skipper and Skipper, 1956a; Wolfsheimer, 1957). The water conditions found to be suitable for the successful breeding of *discus* are summarized in table 1.

TABLE 1

WATER CONDITIONS FOR THE SUCCESSFUL BREEDING OF *Symphysodon discus*

Breeder	Temperature	pH	Hardness (as $CaCO_3$)
Armbruster	85° F.	6.8	?
Dale	81° F.	7.1	142 ppm
Matson	80° F.	7.4	356 ppm
Saphian	80° F.	6.8	50 ppm
Skipper	75°–85° F.	6.2–6.6	65 ppm
Wolfsheimer	79° F.	6.9	68 ppm

What is this important first food of *discus*? Wolfsheimer (1957) supposed that a heavy coating of protective slime developed on the parents and that this supplied the first and only food for the fry. R. Skipper (1956b) thought that the young fed on a particular microorganism that lived commensally on the skin of their parents. I suggested to Skipper that a special secretion by the parental skin might be the source of nourishment and proposed to examine

the skin of anesthetized breeders and adult non-breeders under the microscope—if he were willing to risk the lives of his valuable specimens. In December, 1956, several large specimens of *discus* were anesthetized in tricaine methanesulfonate and the integument was studied (Skipper and Skipper, 1957).

CHARACTERISTICS OF THE SKIN AND ITS MUCOUS SECRETION

The skin and scales of non-breeding adults revealed nothing extraordinary—only the dense melanin pigmentation and scattered guanin crystals which make this species so colorful. A slight mucous coating, especially above the lateral line, was seen under high magnification. Adult breeders in process of rearing young, however, presented an entirely different appearance. Even to the unaided eye, it was apparent that both parents possessed an abundant whitish material over the entire surface of the body. Under the microscope no algae, protozoans, rotifers, or crustaceans were observed on the parents, but a copious mucous secretion with a granular composition covered the entire body including the fins. The secretion was more concentrated dorsally and, when rubbed gently with the finger, it became filamentous. Clumps and filaments of this mucus were readily dislodged into the surrounding water by rubbing the skin. The mucus had considerable cohesiveness and even the larger young had to tug and jerk to remove it from the parents.

When placed on a glass slide and examined at 430× the mucus was observed to be acellular and amorphous and, therefore, undoubtedly a secretion. Several scales with attached skin were carefully removed with a fine curved forceps from the female parent and a non-parental adult and fixed in Heidenhain's SUSA fluid. It should be mentioned that all of these large specimens survived the experimental manipulations without ill effect. The scales were embedded in paraffin and sectioned at 10 mu (figures 1 and 2). The difference between the skins of the parental and non-parental fish is at once evident.

Numerous, large mucous cells are seen in the epidermis of the parental adult, whereas smaller mucous cells are just visible in the non-parental fish. Moreover, the parental skin is much hypertrophied in comparison with the non-parental skin.

DISCUSSION

Although the gross nature of the mucus and the cells responsible for its secretion have now been ascertained, at least several important questions remain to be answered. What is the chemical nature of the *discus'* "milk"? Presumably, the secretion is hormonally controlled, but probably not by the sex hormones since both parents produce it. When, in relation to the time of spawning, is the secretion produced in considerable quantities? Is the feeding by the fry a necessary stimulus for continued production of mucus by the parents? How do some fry manage to survive in the absence of parental

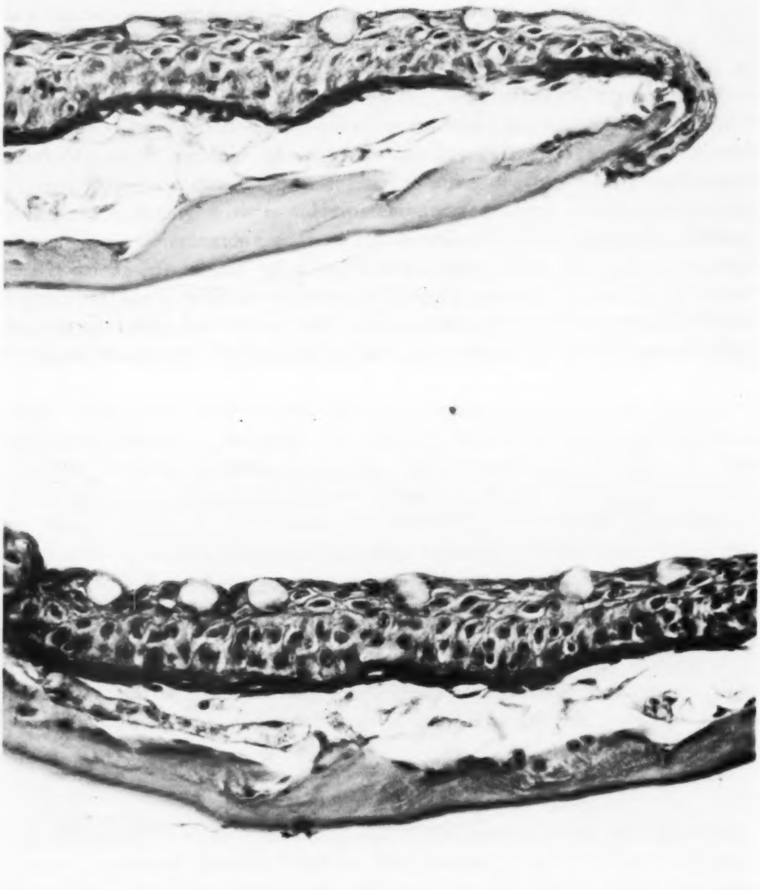

FIGURE 1. Cross sections of skin and scale of *S. discus* from *parental* adult showing epidermal mucous cells and hyperplasia. Compare with figure 2. Hematoxylin-eosin stain.

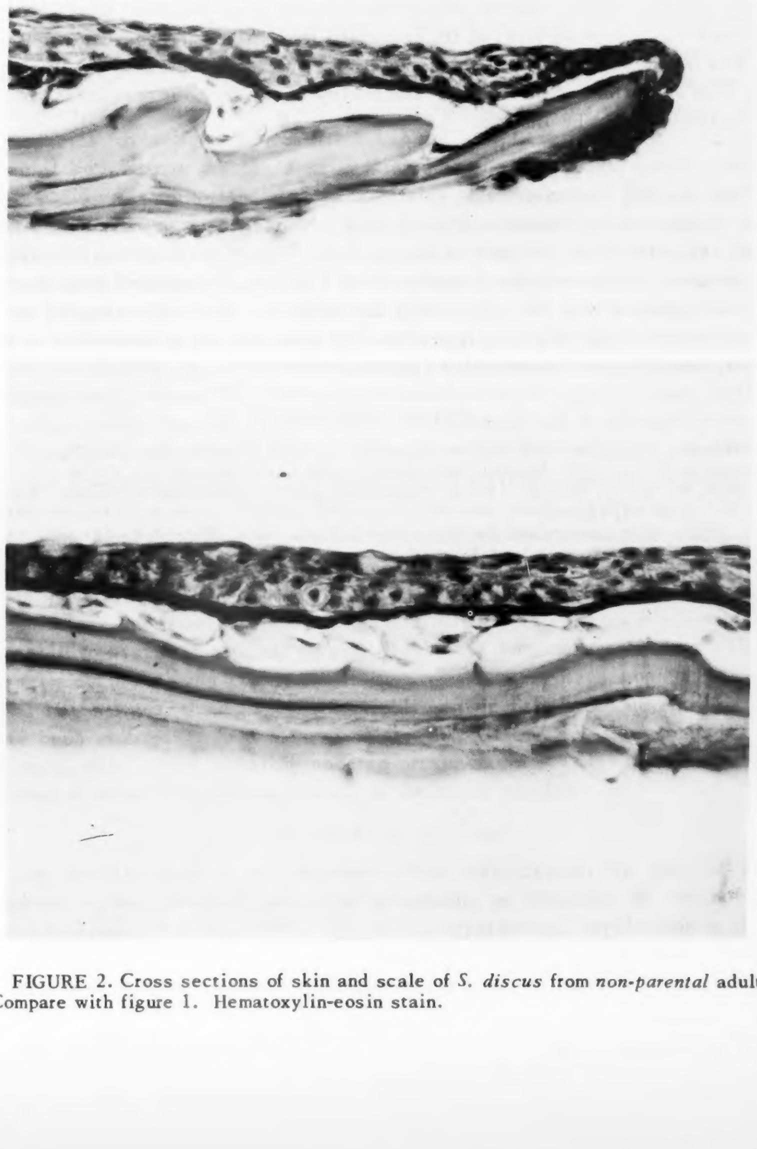

FIGURE 2. Cross sections of skin and scale of *S. discus* from *non-parental* adult. Compare with figure 1. Hematoxylin-eosin stain.

nourishment? Perhaps sufficient mucus is eluted from the parents under certain conditions to sustain offspring kept in the same water in an aquarium without benefit of direct feeding off the parents.

Wild fishes collected from various sources have shown some distinctive color variations. If different Symphysodon species or subspecies exist, this could conceivably account for the apparent differences observed in the nutritional requirements of newly-hatched fry. However, the fishes bred by Saphian and Wolfsheimer (personal communication) were derived from the same parents, so the aquatic environment would seem the more likely basis for survival or non-survival of fry separated from their parents. The mouths of newly-hatched fry may have a peculiar structure adapted to feeding on the skin secretion. If this is true, the ability of the fry to ingest other kinds of nutriment might be limited.

SUMMARY

The amazing parent-offspring relationship in *Symphysodon discus* is probably unique among fishes studied to date. Normally the newly-hatched fry feed exclusively off the skin of the parents. This first food was observed to be an abundant mucous secretion with a granular composition produced by both parents over the entire body including the fins. Histological sections revealed that this food is secreted by numerous, large mucous cells in the epidermis of the hypertrophied parental skin.

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A STUDY OF AN EXPERIMENTAL DROSOPHILA POPULATION IN EQUILIBRIUM

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Most populations of *D. pseudoobscura* have been found to be polymorphic, in nature, with respect to the gene arrangements in the third chromosome (Dobzhansky and Epling, 1944). The polymorphism in these populations is balanced, since it is maintained by adaptive superiorities of inversion heterozygotes over homozygotes (Dobzhansky and Levene, 1948). Experimental populations of this species living in the laboratory usually reach equilibrium due to heterosis of the inversion heterozygotes. This is almost invariably true provided that the flies for the experimental population are derived from the same geographical area (Dobzhansky, 1947; Levene, Pavlovsky and Dobzhansky, 1954). On the other hand, when flies are derived from different geographic populations the results are often indeterminate. Some experimental populations of such geographically mixed origin have also established equilibrium evidently due to balanced polymorphism (Dobzhansky and Levene, 1951; Dobzhansky and Pavlovsky, 1953; Levine, 1955). In other populations the polymorphism disappeared and monomorphism was eventually established. Recently Lewontin (1957) has studied a population formed by mixing adults of two polymorphic populations that had appeared to have reached an equilibrium. He found that the derived population tended to monomorphism. This has raised the question of the stability of polymorphism in populations maintained under constant environmental conditions.

In general, due to the labor of maintaining and sampling experimental populations, most such populations are terminated as soon as it seems reasonable to assume that a stable equilibrium has become established. This usually occurs when a population is from twelve to eighteen months old. No long range study of an experimental polymorphic population at equilibrium has been attempted. The degree of stability of the equilibrium and the changes that it may possibly undergo are unknown. This study was designed to obtain information bearing on the above problem.

MATERIALS AND METHODS

The present study is an outgrowth of an investigation by one of the authors on the effect of genotypic background on heterosis in *Drosophila pseudoobscura* (Levine, 1955). One of the experimental populations of that study was labelled B-4 and at its origin consisted of flies carrying in their third chromosomes the gene arrangements known as Arrowhead (AR) and

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Chiricahua (CH). The AR gene arrangement had been derived from a Californian population, while the CH gene arrangement had been derived from a Mexican population. The rest of the genotype was mainly, if not completely, of Mexican origin. The procedure used to transfer chromosomes of a given geographic origin to a genetic background derived from a different geographic origin is described in the above paper. The methods used to maintain and sample the population are also described there.

TABLE 1

POPULATION B-4, CONTAINING AR CHROMOSOMES OF CALIFORNIAN ORIGIN, CH CHROMOSOMES OF MEXICAN ORIGIN, AND MEXICAN GENETIC BACKGROUND

Month	Per cent CH	Month	Per cent CH
0	80.0	27	33.0
1	74.0	29	31.7
2	68.3	31	42.0
3	62.7	33	30.3
5	59.0	35	24.0
7	49.7	37	34.7
9	49.0	39	...
11	64.0	41	29.7
13	53.0	43	38.7
15	54.7	45	34.0
17	29.3	47	23.0
19	27.3	49	30.0
21	34.7	51	32.0
23	39.3	53	27.7
25	40.3		

RESULTS

Population B-4 was started in April, 1952, and when terminated in September, 1956, was four and one half years old. The observations are summarized in table 1 and figure 1. The population contained initially 80 per cent CH and 20 per cent AR chromosomes. The frequency of CH at first dropped gradually and seemed to establish an equilibrium at about 50 per cent CH, although a significant jump in CH frequency occurred when the population was eleven months old. But for this unexpected rise in frequency of CH at the eleventh month, this cage might have been terminated after fifteen months as having established an equilibrium at the 50 per cent CH level. The unusual rise in CH frequency resulted in the preservation and continued sampling of the population. An unexpected event occurred at the next sampling when the frequency of CH dropped to 30 per cent. For the next three years the cage was sampled every two months (with the exception of July, 1955). It can be seen that the population established a new equilibrium level of about 32 per cent CH.

Some of the departures from this equilibrium, observed in the population after it had apparently reached equilibrium, were nevertheless significant. It should be kept in mind that a departure from the equilibrium such as occurred at the 25th month represents an ostensible increase in CH frequency

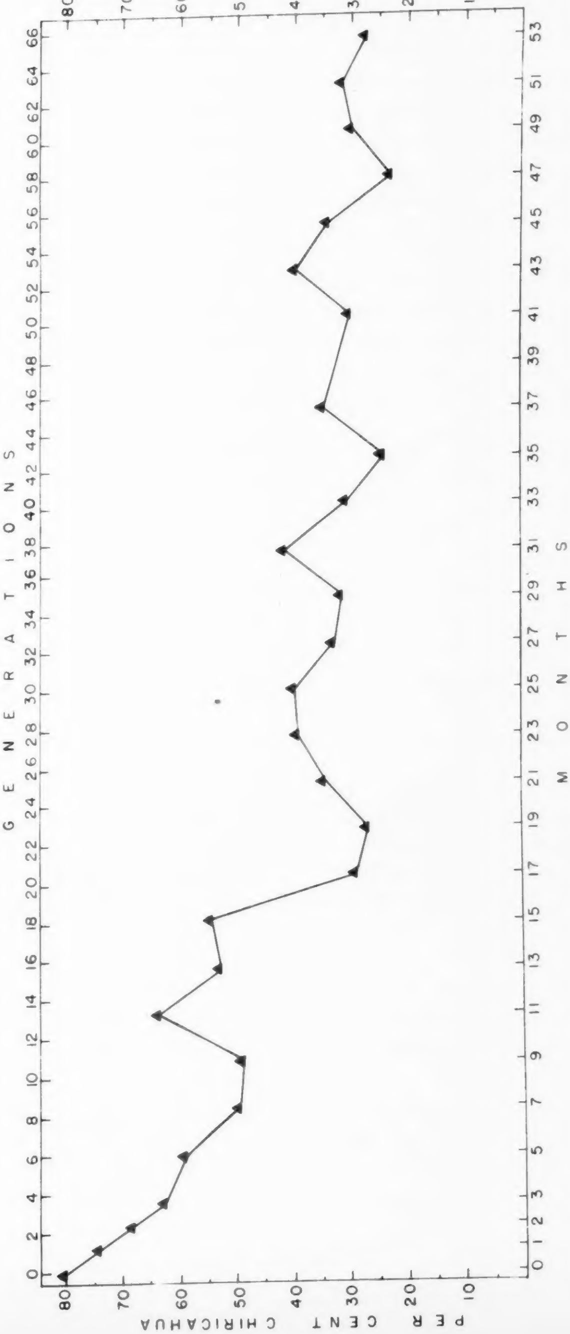

FIGURE 1. Frequencies of chromosomes with the CH gene arrangement of Mexican origin in an experimental population containing AR chromosomes of Californian origin and Mexican genetic background.

of 25 per cent of its equilibrium level, while the data for the 31st month represents a 31 per cent increase in CH frequency above equilibrium. Departures from the equilibrium in the opposite direction have also been statistically significant. The data for the 35th month represents a 25 per cent decrease in CH frequency while the sample taken during the 47th month represents a 28 per cent decrease in this frequency. There is no information available that might explain why the frequency of CH showed such wide deviations from the equilibrium level, only to return to it. It seems remarkable that with such deviations, the equilibrium level was left unchanged after three years. A regression line was calculated for the data covering the three year equilibrium period and was found not to differ significantly from zero. The population had taken some seventeen months to reach its final equilibrium level and had then maintained its composition till the end of the experiment.

EGG LAYING SITES

Recent reports dealing with studies on experimental populations of Drosophila revealed some unexpected ecological complexities in these populations. Previously it had seemed reasonable to assume that a population cage with its cups of food represented a reasonably uniform environment. However in competition experiments between *D. melanogaster* and *D. simulans*, Moore (1952) has reported that the different species preferentially lay their eggs on different parts of the food cup. *D. melanogaster* prefers for oviposition the edge of the food cup while *D. simulans* prefers the center of the cup. Investigators working on DDT resistance in *D. melanogaster* have reported that DDT-resistant flies tend to pupate at the edge of a vial while DDT-sensitive flies tend to pupate at the center of a vial (Sokal and Preston, 1954).

Therefore, in taking the samples during the third and fourth years of this study, we have taken at each sampling some eggs from the center of the food cup and others from the periphery of the cup. Data on the occurrence of the three possible genotypes at the center and at the periphery of the cup are shown in table 2. Comparing these two sets of data for homogeneity by chi-square test, one gets a chi-square value of 1.02 which for two degrees of freedom gives $p = 0.59$. No significant difference in egg laying sites for flies carrying any of these three genotypes is apparent. The food cup apparently represents a single environment, at least with respect to egg-laying sites, for *D. pseudoobscura* flies carrying the AR and CH gene arrangements.

TABLE 2

FREQUENCY OF EGG GENOTYPES DEPOSITED AT PERIPHERY AND CENTER OF FOOD CUPS

Center			Periphery		
AR/AR	AR/CH	CH/CH	AR/AR	AR/CH	CH/CH
307	222	66	294	239	62

DISCUSSION

The finding of long-range stability of chromosomal polymorphism substantiates the conclusions drawn about AR and CH in previous work. Lewontin's findings may not be in contradiction to ours in so far as he used two gene arrangements, namely AR and Pikes Peak (PP), whose initial equilibrium values were roughly 85 per cent AR and 15 per cent PP. Under these conditions it is altogether possible that chance recombinations or mutations may have arisen that favored the more frequent homozygote over the heterozygote and led to the elimination of the less frequent gene arrangement. Attention should be called to an experiment by Dobzhansky (1947) in which he found that some gene arrangements do not form superior heterozygote combinations with all other gene arrangements from the same locality. Since many other gene arrangements are present in the locality from which the AR and PP were taken, it is possible that heterosis between AR and PP has not been firmly established by natural selection. In another paper by Dobzhansky (1957) there is evidence for the loss of PP from populations containing AR and PP in a manner strikingly similar to that found by Lewontin. On the other hand Levene and Dobzhansky (1958) found that an ordinary stock culture containing AR and PP retained both gene arrangements for at least 130 generations with a population size of the order of 40 flies. This may be interpreted to mean that under constant environmental conditions a population consisting of AR and PP will, if ample genetic variance is available, lose PP as progressively better AR recombinants are picked out by natural selection. Under conditions of small population size no opportunity is afforded for this process and the initial superiority of the heterozygote is retained.

The fact that in our B-4 experiment, persistent chromosomal polymorphism was exhibited by a population synthesized from flies of different geographical origins further emphasizes that ample genetic variance and constant environmental conditions do not of themselves produce monomorphism from polymorphism. Although a variable environment may promote the retention of polymorphism, the maintenance of a constant environment does not necessarily mean that monomorphism will supervene since a structural heterozygote may still be the fittest genotype.

SUMMARY

An experimental population of *D. pseudoobscura* was maintained for four and one half years in order to study the stability of its initial chromosomal polymorphism. It was found that it took some 17 months (20 generations) for the population to achieve its final equilibrium frequencies and that the chromosomal polymorphism was then maintained for the remainder of the experiment which was a period of three years (46 generations). It was also found that the different karyotypes of the population did not exhibit any preferences for different parts of the food cups as egg laying sites. The significance of these observations in view of recent findings is discussed.

ACKNOWLEDGMENTS

The authors wish to express their gratitude for the hospitality, encouragement and stimulation given them by Professor Th. Dobzhansky in whose laboratory this experiment was conducted.

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LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

Stilbum zacalloxanthum: TAXONOMY, AND CONIDIAL STRUCTURE AS SEEN BY ELECTRON MICROSCOPY OF THIN SECTIONS

During a laboratory session of a survey course covering the non-vascular cryptogams, a collection of a striking Stilbum, bright yellow in color, was brought in to Dr. I. M. Lamb by one of the graduate students. It was subsequently established in pure culture and found to grow readily on a number of standard media. A subculture was sent for identification to the Division of Botany and Plant Pathology, Ottawa, Canada, but they reported the species unknown to them. In light of this and the following study on the conidial ultrastructure it is here given a specific, identifying epithet and diagnosed:

Stilbum zacalloxanthum Moore, sp. nov.

Synnemata aurea, usque 0.75 mm. altitudine, columnaria vel spathuliformia, singulatim crescentia vel 2-3 congregata, capitulis terminalibus globosis, usque 325 μ latis, mucilaginosis, conidiis repletis. Conidia bacilliformia, hyalina, maturitate $1.5 \times 3.0(-7.5)\,\mu$, monacrogena, sterigmatibus prolata e conidiophoris cylindraceis.

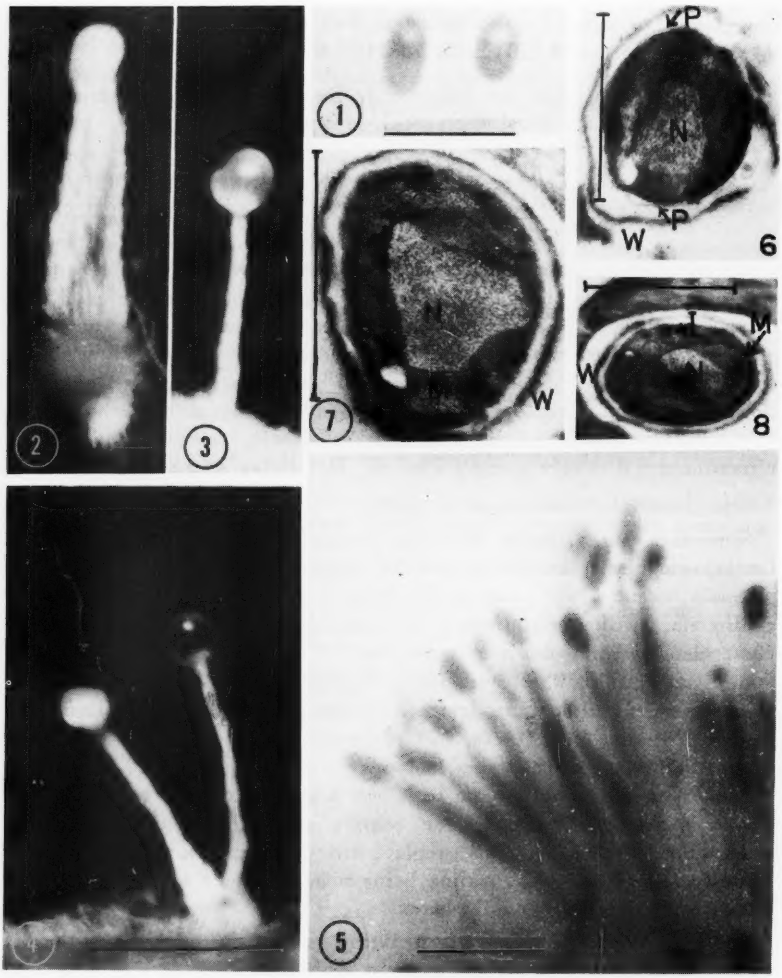
In ligno putrido, West Springfield, Massachusetts, November 1957, leg. Miss Jane Crowell; cultura desicata in Herb. FH Holotypus. Culturae viventes in Herbs. QM, DAOM, BPI et Centraalbureau v. Schimmelcultures, Baarn, Netherlands commissae, Isotypi.

Synnemata bright yellow, up to ¾ mm. high, cylindrical to spathe-like, single or in groups of two or three, bearing terminally an increasingly large mucus drop, up to 325 μ diam., replete with conidia; conidia bacilliform, at maturity $1.5 \times 3.0(-7.5)\,\mu$, hyaline, borne monacrogenously on slender sterigmata from cylindriform conidiophores.

(Etm. ζα-καλλο-ξανθος—very beautiful yellow, in reference to the pigmentation of the synnemata and somatic hyphae.)

This species is placed in this genus rather than the genus Stilbella for the reasons outlined by Mains[7], in which the usurping of the name *Stilbum* Fr. by Lindau for a single Basidiomycete species is held to be counter to the purposes set forth in the International Code of Botanical Nomenclature and the intentions of Fries.

Previous work on the ultrastructure of bacteria (Chapman[4, 5]) led the authors to speculate on the nature of the fine structure of the spores of this fungus whose conidia are within the size range of Bacilli. Portions of agar bearing synnemata were fixed in one per cent $KMnO_4$ in 50 per cent sea water for one hour, washed briefly in distilled water and dehydrated in an ethanol series. At this juncture individual synnemata were isolated and embedded



FIGURES 2-4. Photomacrographs of habit. 2. Young synnema showing spathe-like form and immature sporulating head. Scale line equals 250 μ. 3, 4. Mature synnemata, note the sporogenous regions at the bases of the mucus drops. Scale lines equal 500 μ.

FIGURES 1, 5. Photomicrographs taken using phloxine stain and a blue-green filter. 1. Mature conidia, note lightly staining ?nuclei. 5. Portion of the sporogenous region showing conidiophores and conidia. Scale lines equal 5 μ.

FIGURES 6-8. Electron micrographs of thin sections. 6. The nucleus, N, appears to consist mainly of the low density component; the cell wall, W, and cytoplasmic membrane, P, are readily discerned. 7. The nucleus, N, may be observed to consist of a central, low density zone and three peripheral, higher density zones; the nuclear membrane, M, and cell wall, W, are also prominent. 8. A portion of the amorphous material, A, accompanying the conidia may be seen; one of several cytoplasmic organelles, I, is indicated; M and W represent nuclear membrane and cell wall, respectively; the nucleus, N, here appears as a low density central stratum bounded by two terminal higher density regions. Scale lines equal 1 μ.

in methacryalate at 70° for overnight, thin sectioned, and examined with an RCA EMU-2D electron microscope. The conidia were seen to be embedded in an amorphous mass (figure 8A). Although they resemble bacteria and bacterial spores in size and shape, an examination of their ultrastructure clearly distinguishes them as true karyotic cells. For while they, like the bacteria, possess a cell wall (W), a cytoplasmic membrane (P) and cytoplasmic organelles (I), they differ from the bacteria in the important attribute of possessing a definite nuclear membrane (M). The nuclear content is differentiated into two different classes of material. The lower density and more coarsely granular material appears more centrally disposed while the higher density and more finely granular material usually is arranged in several discrete peripheral zones. The significance of this unusual type of nucleus is uninterpretable at this time. The cytoplasmic organelles appear as membrane-limited structures whose contents are indistinguishable from the cytoplasm. To date there have been few studies of fungi utilizing thin sections in the electron microscope, though there are a number of papers concerned with yeasts[1,2,3,6] and with Penicillium.[8] The interpretation of the included micrographs is, therefore, hampered by a lack of previous data with which to compare them. They are presented here, however, to aid in the accumulation of such data, and in the belief that such observations may eventually provide valuable additional morphological criteria for the understanding of the taxonomic relationships of the fungi and lower plants.

ACKNOWLEDGMENTS

The authors wish to thank Dr. I. Mackensie Lamb, Director of the Farlow Herbarium, for the correction and improvement of the Latin diagnosis.

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PROTECTION OF FOWLS AGAINST THE INDUCTION OF CANCER BY DIBENZANTHRACENE

A question of interest in oncology is whether carcinogens (per se) can induce cancer in animals, or whether they merely prepare the soil (tissue cells) for the seed (template particle of DNA) of an ubiquitous virus, so that it can become incorporated in the enzymic machinery of a normal cell and cause its transformation to a malignant type.

If the latter assumption is tenable, it may be possible to immunize (or non-specifically protect) animals against the development of malignant tumors from carcinogenic stimulation.

An exploratory experiment with a vaccine, based on the above hypothesis, produced significant protection in fowls against the development of cancer from carcinogenic stimulation.

Experiment: Twenty-one Plymouth Rock pullets of the same flock and age (nine months) were maintained under comparable housing conditions and diet (whole wheat and ground wheat mash). Eleven of these pullets were vaccinated and the other ten served as unvaccinated controls. Three weeks after vaccination of the eleven pullets, all 21 fowls were challenged with a potent carcinogen to test the relative immunity of the two groups of pullets to the development of cancer.

The vaccine comprised bacteriologically sterile filtrates (lysates) containing the different strains of live bacteriophage derived from the lysogenic strains of bacteria of the intestinal flora from the 21 pullets. Each strain of bacteriophage was run through serial passages in standard broth medium with young cultures of the intestinal organism which it lysed, in order to obtain a high titre of virulence and quantity of bacteriophage per ml., of lysate (ca. 10^{-10}).

The vaccine was injected intramuscularly in the thighs of the eleven pullets, in amounts of two, four, six and eight ml., with intervals of six days between injections (d'Herelle, 1922). No toxic reactions were noted in the vaccinated pullets. Three weeks after vaccination of the eleven fowls, the 21 pullets were challenged with 1, 2, 5, 6-dibenzanthracene (23.8 mg., of the dibenzanthracene in 0.8 ml., of benzol was injected into each right and left pectoral muscle). (Murphy and Strum, 1941.)

Results: Progressive tumors developed in the fowls during a period from six weeks to six months after injection of the carcinogen. Seven of the eleven vaccinated fowls (63.6 per cent) did not develop cancer or any tumor from the injection of dibenzanthracene, while ten (100 per cent) of the unvaccinated controls and the remaining four vaccinated fowls developed large progressive tumors (fibrosarcoma) with metastases in the lungs or liver. (Murphy and Strum, 1941, found that in a larger group of fowls, 48 out of 53 (90.6 per cent) developed tumors from 1, 2, 5, 6-dibenzanthracene in benzol).

Eleven months after the date of challenge with dibenzanthracene, two of the non-cancerous (immune) fowls were found to be susceptible to the Rous agent when it was injected into their thigh muscles. Autopsy of another of

the non-cancerous (immune) fowls, twelve months after it received the dibenzanthracene, showed the carcinogen to be localized near the site of its injection in the form of an encapsulated pellet. The muscle tissue surrounding it appeared normal, without any gross inflammation apparent.

SUMMARY

In view of the drastic challenge used in the experiment, the protection or immunity produced by the vaccine might be relatively complete against cancer as induced by ordinary (environmental) stimulation. The method used to immunize or protect the fowls may be applicable to other species of animals.

ACKNOWLEDGMENTS

The author thanks Dr. A. Savage, Dr. F. Duran-Reynals for the Rous agent, and Dr. Wm. Boyd for pathological sections of the tumors. Thanks is also expressed to Albert Boswell, Jr., for assistance with the experimental work.

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CYTODIFFERENTIATION: PROTEIN SYNTHESIS IN TRANSITION*

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INTRODUCTION

One of the most fascinating, thought provoking, and yet unilluminated problems in embryology, and in fact, in biology, today is that of the differentiation of cell types from a common precursor. Much of the literature in experimental embryology and either directly or indirectly in related fields of biology is devoted to this phenomenon. In general, however, there seems to be a divergence of interests as related to cytodifferentiation that demands mending. For the most part, the experimental embryologist has been concerned with environmental conditions as they modify cell types, a reflection of more basic chemical differences. On the other hand, the biochemist has lent his energy to exploring the internal machinery of functioning cells. Between these two broad areas of endeavor there exists a field that is lightly trodden. It is the express purpose of this communication to bring together current information from these areas in an attempt to synthesize an account of cell differentiation that is in accordance with observations from such diverse sources.

If we carefully analyze just what cytodifferentiation involves, we must come quickly to the conclusion that the changes incurred during this process arise from changes in the cell protein complement. Structural modifications, the overt signal of differentiation, can only be manifestations of the handwork of proteins, because of the importance of proteins in structure at all levels. Chemical changes are primarily reflections of enzymatic activity, and hence indicate changes in protein concentrations or activities. In the last analysis, then, we can account for cytodifferentiation on the basis of protein synthesis. In the maturing cell this must imply an evolution of protein types in conformity with, and indeed, guiding the direction of the path taken by the cell. The only alternative to the concept of transformation of protein patterns is that a static protein population behaves differentially under varying conditions. This undoubtedly is true to some extent, but cannot account for the known facts concerning differentiation.

We should next consider the cell as a complete unit. By now it should be amply clear to everyone that we cannot consider any cellular component as an entity in itself, except in relation to the pattern of the whole mechanism. Studies on isolated fractions have demonstrated that there is an allotment of

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functions to various moieties of the cell, yet the sum of living processes is invested in the cell as a unit. Studies showing discrepancies in observations, when individual components have been used in comparison to the whole cell, have tended to bear out this conviction. In attempting to account for an evolution of protein species in the cell, attention will be called first to the role of the nuclear constituents. A consideration of cytoplasmic function in this event will be taken up next, followed by the problem of modification of protein molecules under the influence of environmental factors. It is felt that only by tackling the internal mechanisms of the cell as they apply to changing protein patterns, will any definitive concepts of cytodifferentiation emerge.

Since our account of cell differentiation deals with transformations of protein populations, we must describe what is known about the formation of these large polymers as a prelude to consideration of mechanisms of change in their types or concentrations. The specificity and relative homogeneity of protein molecules have, for some time, dictated that their construction is not by fortuitous peptide linkages. We are brought, therefore, to the concept of information theory as it applies to the total formulation of protein species. For this reason, the present paper will concern itself only with those molecules of sufficient size to contain the required "information" for protein specificity. These compounds are deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins themselves.

THE NUCLEUS IN THE DIFFERENTIATING CELL

Aside from the dissenting reports of a few (Ephrussi, 1953; Spratt, 1953; Michaelis and Bartels, 1957) based largely on observations of oöplasmic segregation as outlined by Costello (1948), there is accumulating a very impressive literature on the importance of the nucleus in the process of differentiation. Before proceeding into the role of the nucleus and possible mechanisms of action, we may do well to consider its large molecule moiety in relation to synthetic activity.

1. *Nuclear DNA, RNA and Proteins*

For many years the bearer of genetic information in the nucleus has been considered to be DNA, because of its presence in sperm cells, its constancy in tissues (Mirsky and Ris, 1947a; Chargaff, 1950), its appropriate complexity (Gamow, 1955; Watson and Crick, 1953; Hall and Litt, 1958; Schwartz, 1958), and generic specificity (Chargaff et al., 1950, 1952; Schechtman and Nishihara, 1955). There is some dissension to this concept, however, from Marshak and coworkers (1950, 1953, 1954) who have been unable to detect any DNA in the pronuclei of the echinoderms *Asterias forbesii* and *Arbacia punctulata*. Based on this observation, as well as those of others, they concluded that nuclear RNA is the active agent in cellular synthetic processes, and that it is regulated in its activity by DNA. The recent investigation by Taylor, Woods, and Hughes (1957) has illustrated the dynamic nature of DNA by its uptake of the radiolabelled DNA-specific nucleoside, thymidine.

In 1941 Brachet pointed out the presence of RNA in the nuclei of amphibian eggs. Since this time its presence in chromatin material has been repeatedly verified, and it is particularly concentrated at the region of the nucleolus (Caspersson, 1947; Lettre, 1954), where it is reported to be important in the formation of proteins rich in dibasic amino acids (Caspersson, 1950). Numerous studies on organisms of such diversity as the alga Acetabularia and mammals have consistently shown that labelled RNA precursors are taken up more rapidly by nuclear RNA than by the cytoplasmic portion of this nucleic acid (Jeener and Szafarz, 1950; Stich and Hämmerling, 1953; Barnum et al., 1953; Smellie et al., 1953b, 1955; Allfrey and Mirsky, 1955).

Models of chromosomes based on x-ray diffraction studies (Watson and Crick, 1953) and electron microscopy (Swift, 1953; Sotello and Trujillo, 1958) have pictured the protein moiety in close association with nucleic acid in the form of nucleoprotein. It is generally considered to be contiguous with DNA, but apparently has a similar affinity for nuclear RNA (Caspersson, 1950). Daly et al. (1952), Smellie et al. (1953a), and Ficq and Errera (1955) have reported the presence of radioactivity in proteins of nuclei isolated from animals which had previously been injected with labelled amino acids. Allfrey (1954) with coworkers (1957a, 1957b) and Mirsky (1955) have demonstrated an *in vitro* uptake of amino acids into the proteins of isolated thymus nuclei. Based on experiments using embryonic tissues Waddington and Sirlin (1954) reported a rapid uptake of glycine-1-C^{14} into nucleoproteins of Xenopus larvae and in chick embryos (Sirlin and Waddington, 1956), while Ficq (1953, 1954) found the same to be true for nucleolar protein synthesis in amphibian oocytes. Very recently, Vendrely et al. (1958) have reported a transition in concentrations of basic amino acids between somatic cells and sperm cells in fishes.

We might conclude from the foregoing that the polymers of the nucleus are constantly in a state of flux. To account for nuclear influence on cell differentiation, however, it is necessary to realize a qualitative or quantitative change in such molecules. Indirect evidence of nuclear maturation now exists from the work of Briggs and King (1957) as well as direct evidence of changes in DNA, RNA (see section on *Mode of Action of the Nucleus*) and proteins (Mirsky and Ris, 1947b; Alfert, 1956).

There is evidence from the work of Allfrey (1954) with coworkers (1957b) that nuclear synthesis of proteins is dependent on the structural integrity of DNA, since deoxyribonuclease, but not ribonuclease, destroys or seriously impairs the ability of isolated nuclei to incorporate labelled amino acids into proteins. Unfortunately, we have no such direct proof of the guiding role of nuclear RNA.

2. *The Importance of Nuclear Polymers in Cell Differentiation*

The field of Mendelian genetics is based on the premise that hereditary factors reside in the nucleus. The importance of the nucleus in guiding cytoplasmic form and function has support from Fankhauser (1954), from Danielli (1952, 1955a, 1955b, 1958) in his important work on nuclear trans-

fer in amebae, from Camefort (1958) on the pine embryo, and Hämmerling (1953) working with the alga Acetabularia. In mammals, Gluecksohn-Waelsch (1954) lists examples of hereditary effects in embryonic differentiation, Grüneberg (1957) points out the importance of genes in the development of the axial skeleton, and Markert and Silvers (1955) and Billingham and Medawar (1948) report the importance of genotype in pigmentation patterns.

There is an increasing amount of evidence available from studies on bacteria (Avery et al., 1944; McCarty and Avery, 1946; Lederberg, 1952; Hotchkiss, 1952, 1955) that DNA introduced into cells is capable of inducing cell transformations. Hewer and Meek (1958) have recently observed a similar effect in inducing an intestinal carcinoma in mice. Benoit and associates (1957) have claimed that the injection of DNA from a duck of one strain into that of another, led to somatic changes in the progeny of the recipients. The color of the bills of the offspring somewhat resembled that of the strain donating the polymer. If this experiment could be verified in other laboratories, the results would have profound importance.

3. *Bases for Action of the Nucleus*

a) *Nuclear Maturation.* There has existed for many years the question as to whether there are actual chemical changes in the genetic material of differentiating cell nuclei, or whether a static gene population simply acts differentially under varying conditions imposed by a cytoplasm in transition. Until fairly recently there had been no confirmatory experiments for the former supposition, with the result that the question was left open. Within the past few years, however, reports from several sources have indicated in some animal forms, at least, nuclear maturation is indeed a reality.

Weisz (1951) and Nanney (1956) working with different groups of ciliates have demonstrated nuclear differentiation within the reproductive cycle of these protozoans, such differentiation resulting either in cytoplasmic modifications (Stentor) or in the determination of mating types (Tetrahymena). There have been many reports lately on regional changes in the chromosomes of certain dipterous insects during ontogeny and specifically in certain tissues (Beerman, 1952, 1956; Mechelke, 1953; Breuer and Pavan, 1954, 1955; Taylor, 1954; Rudkin and Corlette, 1957; Stich and Naylor, 1958). Some ascribe the puffing of regions of the giant chromosomes to localized disproportionate synthesis of DNA, while others attribute this to RNA. Such studies have illustrated that the chromosomal material of these animals changes in time during development. The work of King and Briggs (1955) and Briggs and King (1957) have given us proof that the nuclei of frogs are irrevocably modified during early development. Nuclei removed from tissues of post-gastrular animals, on implantation into recently enucleated eggs, direct the development of the frogs in varying degree in the direction dictated by the implanted nucleus. Thus embryos developing from eggs bearing nuclei from endodermal cells, may be grossly deficient in ectodermal derivatives.

The foregoing examples of nuclear differentiation have not taken into account several other relevant observations. A casual glance at various tissues from one organism will reveal that nuclei differ in appearance. These differences, of course, have been used by histologists as diagnostic features of various cell types. Flexner (1955) has reported that the development of these visible morphological characteristics of the nuclei of nerve and hepatic cells is closely tied in with other changes in cell physiology and morphology. Until such time as a cause and effect relationship may be established between these cell features, such an observation can have little more than descriptive value.

b) *Unequal Segregation of Nuclear Components.* Differences in nuclear composition need not arise exclusively through changes in the genetic make-up of *one* cell in time, but may also be brought about by an unequal division of nuclear materials in cell division during the early life of the individual. This might involve whole chromosomes, parts of chromosomes or individual chemical components of them. Lison and Pasteels (1951) have claimed that there is an unequal quantitative segregation of DNA between nuclei of the sea urchin starting at the 8-cell stage of development. It is recently becoming more apparent that the chromosome number in cells of various tissues in animals is not as static as it was formerly thought to be. Rather wide discrepancies in chromosome counts in mammalian liver cells have been revealed. Walker (1957) associates polyploidy in the transitional epithelium of the mouse urinary bladder with the differentiation of these cells. Tumor cells of many types are noted for the inconsistency of their chromosome number, with counts summarized only as averages. Sharma and Sharma (1957 a, 1957 b) have developed a theory for differentiation in plants (where ploidy is not uncommon) based on hypo- and hyperploidy in various tissues.

4. *Mode of Action of the Nucleus*

Given the information that nuclear materials are continuously in a state of flux in the differentiating cell, that the nuclei of some cells of some animals, at least, show morphological changes manifested in general appearance, in chromosome number or in chromosome regions, we must next account for the mode of action of the nucleus during histogenesis.

This could be accounted for, in a superficial way, by describing cytoplasmic changes that are known to have a classical genetic basis, that is, to be under nuclear control. Any account of nuclear influence on cytodifferentiation must deal with its directive action on cytoplasmic events, because it is in the cytoplasm that differentiation manifests itself. For example, Caspari (1955) and Caspari and Blomstrand (1956) have found evidence that the genotype of the mouse determines characteristics of the mitochondria that are detectable cytologically, chemically, and serologically. Weisz (1951) has shown clearly that the presence and function of the cytoplasmic kinetosomes of Stentor are under the influence of the macronucleus. Sonneborn's (1951) work on the Kappa factor further illustrates

the dependence of cytoplasmic particulate matter on genotype. The importance of this dependence has not been diminished by the recent consideration of Kappa as an infective virus (Lindegren, 1957). Sonneborn (1950) and Beale (1952) have both shown the role of the nucleus in the determination of the ciliary antigens of Paramecium.

But such accounts as these tell us only about the end result of nuclear action, and nothing of its immediate specific function. The only truly appealing explanation of the nuclear role in cytodifferentiation deals with its synthetic ability. In discussing this I shall consider the transportation of information in the form of polymers of high molecular weight from the nucleus into the cytoplasmic environment. The importance of nuclear components in cytoplasmic protein synthesis has been treated with concern by Spiegelman (1957).

Lines of evidence of nuclear control over protein synthesis are numerous. Because most of these deal with the mature cell, we can only assume that similar events are valid for immature cells.

Previous comments on nuclear activity suggest that this cellular inclusion is a dynamic one. It has been shown to synthesize all of the types of polymers necessary to account for cell differentiation. Allfrey and Mirsky (1957 c) have given us evidence that *nuclear* ATP, an essential compound for syntheses, is governed in its production by the nucleic acids of the nucleus. The studies on bacterial transduction summarized by Hotchkiss (1955) and on phage infection (Hershey and Chase, 1952) illustrate that DNA, when introduced into the bacterial cell, has the ability to direct protein synthesis. The foregoing examples point out the potential of the nucleus as a structure for synthetic activity.

Genetic studies also afford evidence of the role of chromatin material in cytoplasmic protein formation. Harris and coauthors (1956) state that the specificity in the formulation of insulin is directly controlled by genotype. The arrangement of amino acid residues in the hemoglobin molecule has recently been reported to have a similar guidance (Ingram, 1958). Hemoglobin of individuals with sickle cell anemia has been found to differ from that of the normal person by the substitution of only one amino acid type in the protein strand. The ability of an animal to produce antibodies has a genetic basis, a conclusion supported by studies of pedigrees of humans with agammaglobulinemia. Similarly, Lindegren (1957) has reported that a transferred adaptive enzyme could not be maintained in a microbial system lacking the appropriate genotype.

Evidence of nuclear influence on cytoplasmic synthesis also comes from the work of Lark and Maaloe (1956) who found that in dividing Salmonella cells, DNA synthesis preceded RNA synthesis, which in turn preceded protein formation. Laird and coworkers (1955) found that the sequence of polymers produced in the formulation of cytoplasmic protein in cells of the regenerating rat liver were: nuclear protein, cytoplasmic RNA and cytoplasmic protein. The function of the nuclear components in synthetic activity will be treated shortly.

Enucleation studies performed on Acetabularia by Hämmerling (1953, review) and Stich and Plaut (1958) and on Amoeba by James (1954), Mazia and Prescott (1955) and Ficq (1955) have demonstrated clearly that the synthetic capacity of the cytoplasm is under nuclear control. At some time after enucleation the uptake of nucleic acids in the cytoplasm is markedly curtailed. Chantrenne (1958) has recently discussed the results of such studies.

Finally, the actual visual liberation of materials from the nucleus into the cytoplasm has been described. Most attention has been given to the nucleolus as the organizing center for the elaboration of materials to be ejected into the cytoplasm. Caspersson (1950) was one of the first to report the production of basic proteins in the region of the nucleolus, from which they diffuse radially to the sites of cytoplasmic protein formation. He did not assign the cytoplasmic RNA a nuclear origin, nor did he give any account of the synthetic mechanism. In this connection it is interesting to note that Vendrely et al. (1958) have found a transition in types of dibasic amino acids in the nucleolar proteins during ontogeny. Dodson (1952) described the passage of discrete particles from the nucleolar region of the shark egg into the cytoplasm, such particles acting as centers for the synthesis of yolk. Lettre (1954), Mazia and Prescott (1955) and Duryee (1950) describe a similar movement of nucleolar material into the perinuclear region. In partial explanation of this, De Robertis (1954) has noted that at certain times in the cycle of a cell, chromatin comes into contact with the nuclear membrane.

Many studies have indicated that the nuclear envelope should offer little resistance to the passage of molecules, even at the polymeric level of size. There is general agreement that this membrane is perforated with holes large enough to be seen with the electron microscope (Anderson, 1953; Gall, 1954; Pappas, 1956; Anderson and Beams, 1956; Wischnitzer, 1958).

When we come to the nature of the material elaborated by the nucleus, we are left (as is unfortunately, if not tragically, the usual situation in studies of this type) with much conjecture and very little in the realm of fact. We must, however, on the basis of transfer of information, consider the liberated material to be either (or a combination of) DNA, protein or RNA. The deoxyribonucleic acid may be ruled out immediately, because of its conspicuous absence in the perinuclear space. Mention has already been made of Caspersson's observations of the movement of basic proteins. There are no definitive experiments on the transfer of other proteins, and so our knowledge of the movement of these polymers rests at this point.

Most workers in the field of protein synthesis believe that the carrier of information is RNA. But, again, this is somewhat speculative. There is some evidence for this contention in the work of Goldstein and Plaut (1955) and Prescott (1957) who followed the movement of labelled RNA following nuclear transplantation and enucleation respectively.

Fragments of information suggesting the importance of RNA in protein synthesis, and hence as the probable carrier of information from the nucleus,

include the work of Gamow (1955), who states that this nucleic acid has exactly the required amount of information in its molecular structure for the orderly arrangement of amino acids, assuming that a triplet of bases specifies one given amino acid. Elson and Chargaff (1955) claim common regularities in RNA, a prerequisite to formulating proteins in a non-random manner. Davidson (1953) has reported evidence for organ specificity in RNA within one given species, and at different stages of development. Working with a bacterium, Caldwell and associates (1950) reported that the RNA content of the cell is approximately proportional to the rate at which the cell grows, that is, to the rate of protein synthesis. Further evidence for the importance of ribonucleic acid in cytoplasmic protein formation is provided by the work of Baron et al. (1953), Spiegelman (1955) and Spiegelman et al. (1955) who found that x-irradiation, while inhibitory to the synthesis of DNA, did not affect RNA or protein synthesis to the same degree. Pardee (1954), using nitrogen mustard, obtained the same results. In similar vein, Brachet (1954) on onion root tips, and Landman and Spiegelman (1955) on micro-organisms reported that ribonuclease markedly impaired the production of proteins from amino acid precursors. Spiegelman (1956 a) in conjunction with Simkin and Work (1957 a) stated that when RNA synthesis is blocked, protein formation ceases. It has been this type of work that has led many workers to suspect that RNA is the material that is elaborated by the nucleus. Very recently, Zubay (1958) has proposed a mechanism for the transfer of information from DNA to RNA, based on the premise that the code for RNA bases resides in base-pairs joining the DNA duplex strands.

Further information comes from work on viruses in which Gierer and Schramm (1956); Fraenkel-Conrat (1956), Jeener (1956, 1957), Kozloff and Lute (1957), and augmented by Fraenkel-Conrat et al. (1957 b) have found that the nucleic acid of the tobacco mosaic virus (all of which is RNA) carries the genetic information for its own replication, and for the formation of specific proteins.

Based on the cyclic turnover of intracellular materials, Brachet (1947 a, 1947 b, 1952, 1955) has concluded that RNA, arising in part from the nucleus, is directly responsible for protein synthesis. Using radioactive isotopes, Gale and Folkes (1954, 1955) came to the same conclusion. Thus Smellie and associates (1953 b), Barnum et al. (1953), Stich and Hämmerling (1953) and Allfrey and Mirsky (1955) found that the precursors of nucleic acids are usually incorporated more rapidly into nuclear than into cytoplasmic RNA.

Finally, enucleation studies on *Amoeba proteus* (Linet and Brachet, 1951; Brachet, 1955) and on Acetabularia (Vanderhaeghe and Szafarz, 1955) reveal that enucleated cytoplasm eventually loses the ability to synthesize RNA, such an event leading to a similar loss in ability to form proteins.

There is thus much evidence, mostly circumstantial in nature, to the effect that RNA, produced in the nucleus, is the carrier of genetic information for the cytoplasmic synthesis of proteins. There are no studies to my knowledge that prove or disprove whether the RNA is transported alone or in conjunction with protein (ribonucleoprotein, RNP).

THE CYTOPLASM IN THE DIFFERENTIATING CELL

1. *General Considerations*

While there is little doubt that the nucleus has a direct role in the formation of cytoplasmic proteins, many diversified studies have revealed that the cytoplasm also serves to a very important degree to this end. It is the cytoplasm that contains the machinery required for such synthesis, provides the medium for such events, and, as will be brought out, modifies the final stages of protein formation, thus adding the required specificity.

Enucleation, nuclear suppression and fractionation experiments have demonstrated that net protein synthesis occurs without the immediate directive influence of the nucleus. Thus Hämmerling (1934), Brachet and Chantrenne (1951), Vanderhaeghe (1954), Brachet and coworkers (1955) and Stich and Kitiyakara (1957) have shown that an enucleated Acetabularia continues to form proteins for a period up to two weeks following surgery. Obviously all of the machinery required is present in the region of the cytosome. Malkin (1954) found that non-nucleate fragments of sea urchin eggs could incorporate amino acids into proteins. This apparently is also true for the eggs of the newt, Triton (Tiedemann and Tiedemann, 1954). Studies on the rabbit reticulocyte have revealed that this cell, on losing its nucleus, is still capable of net protein synthesis (London et al., 1950; Borsook et al., 1952; Koritz and Chantrenne, 1954). Similarly, Jeener and Jeener (1952) and Cohen and Barner (1954, 1955) have been able to demonstrate the ability of thymidine-less mutants of *E. coli* and *T. acidophelus* to synthesize proteins in the absence of thymidine. Many studies on the uptake of amino acids by cell fractions have shown that protein synthesis occurs most rapidly in association with cytoplasmic particles (Borsook and associates, 1950; Hultin, 1950, 1955; Siekevitz, 1952; Keller et al., 1954; Zamecnik et al., 1956; Hoagland et al., 1956; Simkin and Work, 1957b; Chantrenne, 1958).

2. *Cytoplasmic RNA*

Much attention has been given to the location of RNA in the cytoplasm, in view of its apparently eminent part in cellular synthetic activity. Brachet (1950) has treated the cyclic nature of the nucleic acids and Jones-Seaton (1950) has followed the distribution of RNA in the rodent egg during cleavage and the period up to the primitive streak stage. In mature cells most RNA is located in the microsome fraction (Schneider, 1947; Barnum and Huseby, 1948; Hultin, 1955; Palade and Siekevitz, 1956). There is some present in the mitochondria, and Hoagland et al. (1958) recently report a soluble RNA moiety active in protein synthesis.

A previous section in this paper was concerned with the transfer of RNA from nucleus to cytoplasm. Experiments on diversified cells, however, have revealed that much (perhaps most) of the cytoplasmic RNA forms *in situ*. Unlike the condition found in amebae, there is a net synthesis of this compound in the cytoplasm of Acetabularia following enucleation, for a period

roughly equal to that of protein formation. Kruh and Borsook (1955) found that rabbit reticulocytes were still capable of producing ribonucleic acid after they had lost their nuclei, thus paralleling their protein synthesizing capacity. Similarly, Smellie et al. (1953a) and Logan and Smellie (1956) working with rat liver cell fractions and Barnum et al. (1953) on a mouse mammary carcinoma came to the same conclusion, namely, that much RNA has a cytoplasmic origin.

There are several interesting reports arising from studies on *E. coli* (Pardee and Prestidge, 1956; Gros and Gros, 1956) and on yeast cells (Schmidt et al., 1956) that a short period of protein synthesis is necessary prior to RNA synthesis. Munro and Mukerji (1958) state that the amino acids glycine, leucine and methionine have ameliorative value in offsetting RNA depletion in the liver cells of protein-starved rats. These observations are of great importance, if we consider that the template (see below) for amino acid binding is a nucleoprotein, rather than simply RNA.

3. *Cytoplasmic Protein*

The polymers of perhaps greatest concern to the student of cytodifferentiation are the proteins. As far as we know to the present moment, the nucleic acids function primarily, if not exclusively, as templates for the formation of more nucleic acid or proteins. Presumably it is the behavior of the polypeptide chains, whether it be in structure or function, that gives the cell its unique characteristics.

As mentioned earlier, cell differentiation must result from an evolution of types or concentrations of proteins. The field of immunochemistry has revealed very conclusively that such changes in proteins do occur during ontogeny. Thus Flickinger and Nace (1952) and Spar (1953) working with amphibians, Kavanau (1953, 1954) on the sea urchin, and Nace (1953) on the chick embryo, have detected progressive changes in serologically-detectable proteins. This work has been adequately summarized by Nace (1955). The appearance of tissue-specific proteins has been detected by Ebert (1952) for heart muscle, and Flickinger, Levi and Smith (1955) for the lens of the frog eye.

Using chemical means, numerous workers have been able to show changes in enzyme patterns in embryonic tissues. Without specifying here the enzyme systems or organisms used, the work of Sawyer (1943), Moog (1944, 1952), Boell (1946), Karczmar (1955), Fraser (1956) and Davidson (1957) lends further support to the concept of protein change in association with cell differentiation.

CYTOPLASMIC PROTEIN SYNTHESIS

Much importance has been assigned to the proteins in cell differentiation. To account for the changes in protein populations, either qualitatively or quantitatively, it is necessary to account for their formation, and then attempt to find reasons for such changes.

1. *The Importance of Ribonucleic Acid*

Several aspects of the important role of RNA in protein synthesis have already been considered in regard to the suspected agent liberated by the nucleus. In addition to these there have been several studies not dealing directly with nuclear influence. Thus Hultin (1955), Simkin and Work (1957 b) and Chantrenne (1958) have found that proteins are formed most rapidly *in vivo* in association with cell microsomes. Similarly, Borsook et al. (1950), Siekevitz (1952), and Keller, Zamecnik and Loftfield (1954) have found that amino acids are incorporated into proteins most rapidly in the microsome fraction *in vitro*. The importance of RNA in formulating proteins of the mitotic apparatus has been discussed by Stich and McIntyre (1958).

2. *Template Hypothesis*

The non-random nature of the sequence of amino acids in proteins has been appreciated for many years. The precision in pattern of these polymers has led many workers to the assumption that there must exist a directive influence. Haurowitz (1949, 1950) first set down a theory that the information for such precise synthesis was invested in a template, which he considered to be of protein nature, with amino acids arranged in the same order as laid down in the newly synthesized product. This idea has caught the fancy of numerous experimenters, and has formed the basis for much research. Thus, Lipmann (1954), Borsook (1955) and Beale (1958) have reported the formation of proteins at the face of cytoplasmic templates.

Since the time of Haurowitz' original articles on the topic, interest has shifted from protein to ribonucleic acid as the template. Dounce (1952) strongly indicated the importance of RNA in this function, and this has been repeated since by Koningsberger and Overbeek (1953), Hershey (1954), Lockinger and De Busk (1955), Schwartz (1955), Brachet and Ficq (1956), Borsook (1956), and Hoagland et al. (1958). On the basis of numerous types of experimentation, there seems to be little doubt that RNA serves in this role in protein synthesis.

In recent years, however, the value of protein in template function has come to be more obvious. Working with deoxyribonucleoproteins, Korngold and Pressman (1952) found that maximum antibody purification could be obtained by absorption of serum on the residual chromosome fraction which remained after most of the DNA has been removed. Work on viruses by Fraenkel-Conrat and Williams (1955), Hart and Smith (1956), and Fraenkel-Conrat and Singer (1957 a) has revealed that while both viral RNA and protein individually are quite inert, a combination of them as RNP was highly infective. Recently, Hayashi (1958) has found that the inductive capacity of pentose nucleoprotein is impaired by treatment with pepsin or trypsin. Zamecnik and coworkers (1956), Schweet and Owen (1957) and Novelli and De Moss (1957) consider RNP to be the template. Novelli states that structural barriers in the template rule out the possibility of RNA alone acting in this capacity. Again we might mention the observation of Schmidt et al.

(1956) and Gros and Gros (1956) that a period of protein synthesis must precede RNA and protein formation.

3. *Mechanism of Synthesis*

One of the fields in which there has been rapid advances in recent years is that of protein synthesis. Within the past few years this biochemical problem has been lifted from the realm of pure speculation and now rests on rather secure ground. There are still several disputed points, but thanks to the great labor and much imagination of such workers as Dounce, Borsook, Novelli, and Zamecnik these points are of minor nature.

The first step in the formation of protein polymers is considered to be the activation of the amino acids (Pinsky and Stokes, 1952; Zamecnik and Keller, 1954; Hoagland et al., 1956; Zamecnik et al., 1956; Novelli, 1958; Davis and Novelli, 1958) by high energy phosphate bonds. Steinberg and Anfinsen (1952), however, believe that some preformed peptides exist in the cell. The second step is believed to be the arrangement of the activated amino acids on the nucleoprotein template. An intermediate step has been introduced by Zamecnik et al. (1958) and Hoagland et al. (1958) involving the formation of an amino acid-RNA complex prior to transfer to the RNP particles. On the template, condensation occurs in the formation of peptide bonds between the amino acids. There is some doubt as to precisely what happens at this point. Borsook (1956) believes that the proteins are then peeled away *in toto*. He does not think (Borsook, 1953) that simpler peptides are formed because impossibly large numbers of them would be needed. The work of others tends to bear out this opinion. There is, however, the suggestion from the recent work of Anderson (1958) that units simpler than whole proteins may be produced on the template face. Zamecnik and associates (1958) think that the final stage in protein synthesis occurs at lipoprotein interfaces of endoplasmic reticular membranes (see Schmitt, 1957), where cross linking and patternization is completed. Evidence for the last step is admittedly scanty.

The formation of proteins is known to occur primarily in association with the microsomes, that fraction of the cell containing the highest proportion of RNP. Other portions of the cell are also known to play a vital part, however, in this synthetic activity. The importance of mitochondria in providing energy has not been overlooked (Peterson and Greenberg, 1952; Siekevitz, 1952; Beyer and Kennison, 1958). The work of Green (1958) has revealed remarkably well the arrangement of oxidative enzymes in these structures. There is also some evidence that protein synthesis may occur at the mitochondrial surface (Nakano and Monroy, 1958). The final step in protein formation points out the importance of membrane interfaces in the endoplasmic reticulum. Such membranes have been recently reported by Siekevitz and Palade (1958) in hepatic cells, by Rajam and Jackson (1958) and by Beljanski and Ochoa (1958) in bacteria. The last named authors state that such membranes have the ability to couple amino acids.

The assumption is generally accepted that template action is catalytic in nature (Hershey, 1954; Dalgliesch, 1957).

MODIFICATIONS IN PROTEIN FORMATION

Up to this point we have discussed only the mechanism of protein formation without considering factors that may influence such a process in time. Since the crux of the problem of cytodifferentiation involves changes in protein species, we must give due thought to environmental factors, in the broad sense, which may elicit modifications in the qualitative or quantitative production of such polymers.

1. *The Stimulus*

We may first consider the stimulus for the elaboration of proteins. There are two fields of study that may be informative in this respect, namely, the formation of induced enzymes and antibody production. The latter may be thought of as one group of induced proteins, and indeed, recent studies have indicated much common ground in the formation of both. The synthesis of induced proteins of both kinds require the presence of an inducer, energy, and ribonucleoprotein.

In the formation of adaptive (induced) enzymes most studies have been made on bacteria or yeasts, although Knox and Mehler (1951) and Gordon (1952) have described such activity in the liver of mammals and chicks respectively. Halvorson and Spiegelman (1952) and Gale and Folkes (1953, 1954) have reported that pyrophosphate energy and a supply of free amino acids are required.

Since the earlier studies on the inductive formation of enzymes (Monod, 1947; Spiegelman et al., 1947), much attention has been given to the role of the inducer in synthetic processes. Instead of finding a nice, simple configurational relationship between substrate and formed enzyme, workers have discovered that the substrate *may* or *may not* act as the inducer (Monod, 1956). Thus, Spiegelman and Halvorson (1954) have found that alpha-methyl glucoside, an analogue of maltose, can induce the synthesis of maltase in *Sacharomyces cerevisiae.* They believe that the substrate does not combine with the maltase in the process of stimulating the synthesis of this enzyme. Monod et al. (1951) have reported that a substance with no demonstrable affinity for the enzyme being formed, may nevertheless induce the formation of the enzyme, while a substance with a high affinity may have no inducing activity. On the basis of their work, these authors concluded that the affinity of the substrate for enzyme is unrelated to the ability of the inducing substance to promote enzyme synthesis. Adding confusion to the picture, Hebb and Slebodnik (1958) have found that the aeration of anaerobically grown yeast in a glucose-buffer solution induces the synthesis of two enzymes associated with mitochondrial function. Moreover, Chantrenne (1956) states that the action of the inducer seems to be related to the production of new RNA, which is specific for the synthesis of the induced enzyme. The foregoing account will reveal that at present, at

least, the action of the inducer in protein synthesis is about as nebulous as that of the "evocator" in embryological parlance.

Reference has been made previously to the abolition of induced enzyme synthesis under the influence of ribonuclease and base analogues. This has been confirmed by Gale and Folkes (1953). It might be mentioned in passing that a similar inhibition of enzyme synthesis has been reported in the presence of glucose (Neidhardt and Magasanik, 1956) and amino acid analogues (Halvorson and Spiegelman, 1952).

Spiegelman's (1948) original concept of induced enzyme formation embodied the idea of self-reproducing cytoplasmic units (plasmagenes) acting as templates to account for the sigmoid-type curve he obtained for enzyme synthesis. This was in contrast to the earlier hypothesis of Yudkin (1938), and later supported by Mandelstam and Yudkin (1952), that the formation of proteins followed the mass action principle. If so, the accumulation of enzyme, according to Spiegelman (1946), would demand a hyperbolic-type curve, which, indeed, was inconsistent with his observations. This conclusion seems unwarranted to this writer, because it would be the concentration of *unbound* (inactive) protein which would determine the rate. Indeed, the mass-action principle in itself could act in a regulatory manner, with the concentration of unbound enzyme being a function of the substrate at hand. The most sound work indicates, however, (aside from actual liberation of the enzyme from the template) that there is a linear time course in the formation of induced enzymes (Ephrussi and Slonimski, 1950; Porter et al., 1953).

The importance in such studies on induced enzyme synthesis rests with the knowledge that their formation may be stimulated by substances acting as inducers. Precisely how they work is not known, but it is clear that they regulate, in some manner, the final form of the protein in preparation for its normal function. Vogel (1957) has set down a satisfactory explanation of inducer and repressor action. His account, in reasonably good accord with our present knowledge, postulates that the cell already has the information to produce a given enzyme before the entrance of the repressor or inducer. Accordingly, it is the function of such substances to participate in the 1) separation of the enzyme from the template, and 2) final shaping of the enzyme. At least the second function seems reasonable.

The concept that the cell possesses the information to produce a certain protein prior to the admission of a stimulating substance has support from studies on antibody production. As we know, guinea pigs have only limited ability to produce antibodies. The same situation is found in humans with agammaglobulinemia. We interpret this to mean that the cells of such individuals have lost the code for such protein synthesis even in the presence of the inducer, and that this loss can be traced back to the nuclear polymers.

Again, as for induced enzyme formation, the formation of antibodies requires energy and free amino acids (Green and Anker, 1954). The work of Taliaferro (1957) has indicated that the antibody-synthesizing mechanism is formed during an induction period characterized by being x-ray sensitive,

and, hence, believed to involve RNA or DNA. Once the mechanism has been established, formation of antibodies occurs rapidly using only amino acids as precursors. According to Fagreus (1948) and Thorbecke and Keuning (1953) the cell has sufficient integrity (information) to produce antibodies *in vitro*, provided the animal had been previously sensitized. The recent work of Nossal and Lederberg (1958) suggests that a cell, when stimulated by two contrasting antigens, will produce antibodies directed primarily at one, or the other, antigen.

2. *Microheterogeneity of Proteins*

But perhaps the most significant information to come out of studies of this type is that the antibodies produced show considerable heterogeneity with respect to total configuration (Haurowitz, 1956; Putnam, 1956; Talmadge, 1957; Nisonoff and Pressman, 1958) and to reactive sites (Kabat, 1957). Both Haurowitz and Talmadge acknowledge that the discrepancies do not occur in discrete steps, but rather, there exists a wide, continuous spectrum of protein molecules produced in response to the presence of one antigen. From such studies has emerged the concept that biological activity is not dependent on invariant structure. As in so many (if not all) biological phenomena, there is functional latitude. Similarly, Spiegelman (1956 b) has found considerable heterogeneity in induced enzymes, although he thinks that the changes are discrete, resulting from their formation at different cellular sites. Fox (1953), after working on protein degradation, has likewise come to the conclusion that proteins are not homogeneous. He prefers to call a protein a family of molecules. On the basis of these studies we might consider the data given for a particular protein as being those for the "average" molecule of the protein.

3. *Possible Mechanisms of Protein Transition*

We have come finally to an account of changes in intracellular protein patterns, which I have proposed is the basis for differentiation. We have, at the same time, come to the end of substantiating facts, and must resort largely to conjecture. This is indeed unfortunate, but it does point to the direction in which most embryological investigation needs be slanted. Actually it is in this field that much research has been performed, but in such a superficial manner, that little basic information on causes of cytodifferentiation has arisen. As examples, the inhibitory studies of Ebert (1950, 1952), Duffey and Ebert (1957) and Rose (1952, 1955, 1958), the experiments bearing on the whole field of induction (Holtfreter, 1934, 1944; Nieuwkoop, 1952; Grobstein, 1955 a; and many others), studies on tissue mass relationships (Lopaschov, 1935; Chalkley, 1945; Grobstein, 1952, 1955 b) and, in general, all embryological investigations dealing with cell-cell interactions (Spratt, 1954) including hormonal effects, have yielded valuable information on cell differentiation, but none of them has attempted to tie in any environmental effects with the basic machinery responsible for differentiation. It is my belief that any "break-through" in the field of

embryology will relate changing environmental conditions with changes in internal synthetic mechanisms.

We can, however, relate what scanty information is available to us on this problem, and then speculate on points in the protein-synthesizing apparatus where changes may occur, giving evidence where possible.

The radiation cytologists know that DNA is particularly susceptible to damage from ionizing radiations and certain toxic substances such as mustards, etc., but in the normal cell the influence of such agents may be presumed to be negligible. We do know, however, as discussed here under *Nuclear Maturation* that certain chromosomal changes do occur during embryogeny, some of which have been shown to be of an heritable nature. There is a little information available on structural modification of nuclear nucleoprotein, as seen in the work on localized puffing of chromosomes in certain dipterous insects, and on changes in the nucleolus (references cited previously). The nucleus, then, presents two compounds in which structural modification of the protein-synthesizing apparatus may be altered, namely, DNP or RNP. Whether changes in RNP are consequences of modifications in DNP is conjectural, although this is suspected (Zubay, 1958).

In the cytoplasm there also exist several points subject to alteration by environmental influence. Cytoplasmic RNA has been shown to change in configuration under the influence of changes in ionic strength of the surrounding medium and by ribonuclease activity (Dickman and Ring, 1958). Roth (1958) has recently found an inhibitor in the cytoplasm which influences the activity of RNase, which in turn can modify RNA. The inhibitor is itself subject to alteration by ionic strength and pH of the medium. We can thus visualize, in a cursory manner at least, how some structural alterations in the template may be brought about. In addition to this, the work on inducers would lead us to believe that the substances may, in some, as yet vague manner, modify the template. The work of Koppel et al. (1953), Bieber (1954), Waddington et al. (1955) and Liedke, Engleman and Graff (1957) has indicated the importance of structural analogues of nucleic acid precursors in inhibiting protein synthesis, reflected in the distorted differentiation of cells. As pointed out, the inducing substance may also modify the protein as it is liberated from the template. Certainly we know from the work of Doty (1956), and many others, that proteins are very susceptible to environmental conditions for their form and activity. As it is visualized, changes in protein synthesis may occur in the cytoplasm as a consequence of modification of the RNP template or final protein configuration under the influence of environmentally located factors. Template modification may, of course, also be a result of changes in structure of the nuclear polymers.

In parting, mention of the importance of intracellular substances in modifying protein formation will be given. We have no reason to believe that substances normally found in the cytoplasm cannot act as inducers or repressors in protein synthesis, in the same manner that such substances do when introduced into the cell. Thus, normal cell contents may presumably direct protein synthesis in a manner similar to inducers of induced protein

synthesis or to antigens. We might well expect that the degree to which they are capable of such modification will be a function of their concentration. It has been shown by the work of Lwoff (1946), Potter and Heidelberger (1950), Recknagel and Potter (1951) and Williams (1951) that a given metabolic reaction may initiate and sustain, to variable degrees, a secondary, interlocking reaction. In other words, the end product of one reaction may act as an inducer or repressor for protein synthesis that will lead to a secondary reaction. We might picture the transition of enzyme systems, and in fact all proteins, as the result of the development of substances arising from a reaction, which in turn call on other reactions as a consequence of enzyme synthesis. The nature (quality) and concentrations of reactants will therefore determine the route which the cell will take, guided by limitations imposed by the nucleus in the form of possible RNP structure, in its differentiation. The ideas of Rusch (1954) are noteworthy in this connection.

In such a scheme the role of oöplasmic segregation of many diverse materials assumes a prominent position, and needs no further amplification. Moreover, we can account, in a more precise way, for inductive influences at all levels by considering the effects of introduced materials on the protein synthetic apparatus of the cell. I do not think that we can continue to talk about cell differentiation much longer without discussing causal mechanisms. The sooner we rid our laboratories of such truly uninformative terms as "evocators," "competence" and "modulations," etc., and replace them with operational terms, the better off we will be.

THEORY

The following theory, based on observations and some speculation previously outlined, is designed to account for cytodifferentiation as a consequence of changes in cell protein composition. No verification, other than what has already been encompassed in the text of this paper will be offered at this time. Figure 1 will serve as a diagrammatic model of events.

We might picture the DNA as the definitive code for protein formation in the cell. In conjunction with protein it is capable of acting as a template for transfer of information to nuclear RNA. Some of the RNA is bound to the chromosomes with protein, as RNP. Moreover, it is believed that some free RNA is formed within the nucleus, but whether it is formed from DNP or RNP is not known. This point is of little consequence in our discussion of cytodifferentiation, except as a possible site for modification in the code.

The next event is the movement of free RNA (or RNP?) to the cytoplasm where RNP is produced. The template thus formed has limited ability to reproduce itself. In the cytoplasm most of the RNP becomes part of the microsomal fraction of the cell, where most protein synthesis occurs. On the face of the microsomes the RNP, acting as templates, and aided by other cytoplasmic constituents, elaborates proteins. The configuration of the proteins thus produced will be somewhat a function of the template form, and somewhat a function of other environmental factors, such as pH, ionic strength, and the nature of the inducing substance.

Based partly on observations and partly on conjecture we may imagine that alterations in protein form, and hence activity, may result from environmentally inflicted changes at a number of sites in the protein-formulating mechanism. DNA itself may be modified. The RNA resulting from it may also be changed. The replication of RNA is another possible source of alteration. Finally, the proteins which emerge from the template face are subject to environmental effects. These positions, where modifications in the protein-forming machinery may take place, are indicated in Figure 1 by numbers. The changes in protein molecules thus manifested will form the basis for chemo-differentiation, a concept familiar to us all.

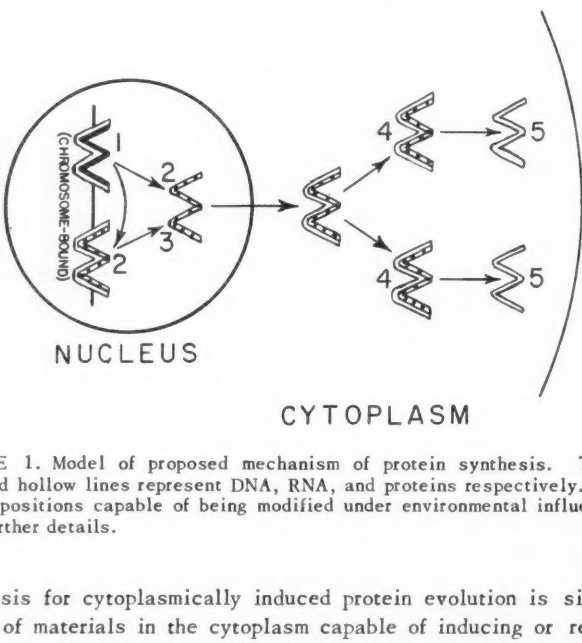

FIGURE 1. Model of proposed mechanism of protein synthesis. The solid, barred, and hollow lines represent DNA, RNA, and proteins respectively. Numbers represent positions capable of being modified under environmental influence. See text for further details.

The basis for cytoplasmically induced protein evolution is simply the presence of materials in the cytoplasm capable of inducing or repressing protein synthesis. The unequal distribution of egg materials through segregation, and the introduction of materials (induction) will both influence and direct the final protein population. Such change may be manifested in the DNA of the cell, and hence may be of an irreversible nature. The embryologist would then say that this cell is "determined" in a certain respect.

Differentiation is thus pictured as an evolution of functional and structural proteins of a cell resulting from cytoplasmic components modifying the protein-synthesizing mechanism. The spectrum of change will be determined, therefore, both by the cytoplasm and by the mechanism which has its origin in nuclear DNA. When an equilibrium has been reached for the particular cell such that protein formation is relatively stable, we say that the cell is then mature.

SUMMARY

A theory has been extended which accounts for cytodifferentiation as the consequence of an evolution of structural and functional protein species. The formation of proteins has been treated with consideration given to the roles of nuclear and cytoplasmic components. Possible mechanisms for the alteration in quality and quantity of proteins has been sought, in an attempt to account for the known facts about histogenesis.

The theory postulates that, within the limits imposed by the nucleus on the cytoplasm in the form of possible ribonucleoprotein template configurations, the quality and concentrations of diverse cytoplasmic substances, acting as inducers or repressors, guide the cell along its path to differentiation. Cell maturation is reached when there is some semblance of stability in the interaction between reactants and the protein-synthesizing mechanism at various levels, including the nucleus.

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